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**Superhelical stress restrained in plasmid DNA during repair synthesis initiated by the UvrA, B and C proteins *in vitro***

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**ABSTRACT**

Purified UvrA, UvrB, UvrC, UvrD, PolA and Lig proteins from *Escherichia coli* have been used to assess the effect of nucleotide excision repair on the conformation of native negatively supercoiled plasmid DNA in an *in vitro* test system. The analysis of labeled reaction products on specific gel systems suggests that the Uvr excinuclease has the ability to restrain the superhelical stress in the template DNA during the repair process. This feature, observed in the case of the Uvr system is not found if the repair reaction is initiated by T4 endonuclease V or *Micrococcus luteus* UV endonuclease.

**INTRODUCTION**

Nucleotide excision repair in *Escherichia coli* is mediated by a multi-enzymatic system involving the UvrA, B, C and D proteins as well as DNA polymerase I and ligase. *In vitro* experiments have shown that these 6 proteins are necessary and sufficient to perform the complete nucleotide excision repair reaction (1,2,30). The most striking feature of the Uvr repair system is its very broad substrate specificity, acting on purine and pyrimidine damage, monoadducts, diadducts, dimers and crosslinks, helix stabilizing and helix destabilizing adducts. The recognition of a large number of different DNA adducts seems to be a general rule for excision repair pathways as identical observations have been made in other organisms including yeast, *Drosophila*, rodents and man (3). Hence it has been suggested that this specific repair system does not recognize the DNA adduct *per se* but rather a distortion in the DNA double helix (4). Recent experiments with purified UvrA, B and C proteins seem to confirm this model. DNase I footprinting has revealed that the Uvr excinuclease covers an area of approximately 19 base-pairs around the DNA adduct and that the non-adducted strand seems to be protected more efficiently against DNase I than the adducted strand (5). This finding is in agreement with the observation that a pyrimidine dimer in an specific Uvr protein-DNA complex is still accessible to *Escherichia coli* photolyase (4).

The wide substrate specificity of nucleotide excision repair seems *a priori* to be mainly advantageous as the organism can respond to a variety of environmental hazards with the use of just one enzymatic repair system. However, the fact that the DNA adduct is not recognized directly by the repair system implies that the simplest and most secure form of DNA repair, namely direct reversal of the damage in a single step as found for other repair systems (e.g. photoreactivation, methyltransferase) is not possible. Instead the Uvr excinuclease attacks the DNA backbone which results in the excision of a 12-13 bases long oligonucleotide containing the

DNA adduct (6). This incision of the DNA backbone again might have profound implications on the structure and conformation of DNA in the cell. The native bacterial DNA double helix is partially underwound *in vivo* which results in the formation of a negative superhelical structure (negative supercoiling). Recently it has been shown that RNA polymerase plays an active role in DNA supercoiling and that the fine tuning of the degree of superhelicity is regulated by DNA topoisomerase I or DNA gyrase (7). Part of this DNA superstructure is stabilized by proteins in nucleosome-like structures (8), however, a significant part of the *Escherichia coli* chromosome contains unrestrained negative supercoils (9,10). Incision of the DNA backbone in such unrestrained areas could lead to extensive relaxation of a large area of the bacterial chromosome. As DNA supercoiling plays an essential role in cellular regulatory processes (11) the introduction of nicks into the DNA backbone by DNA repair could have a major impact on the physiological state of the cell.

In this communication we have used an *in vitro* test system containing highly purified UvrA, UvrB, UvrC, UvrD, PolA and Lig proteins to analyse the fate of negatively supercoiled plasmid DNA during nucleotide excision repair. Our results suggest that UvrABC mediated excision repair has the ability to restrain supercoiling in the plasmid molecule during the repair process.

### MATERIALS and METHODS

#### 1. Proteins and Enzymes

The purification of the UvrA, UvrB, UvrC and UvrD proteins has been described previously (12). Purified PolA and Lig proteins from *Escherichia coli* were purchased from Pharmacia. T4 endonuclease V was isolated from an overproducing strain harbouring plasmid p<sub>tac</sub>-denV (a gift from Dr. J.K. de Riel, Philadelphia) and was purified as recently described (13). All proteins were judged to be more than 95% pure after analysis on Coomassie stained SDS polyacrylamide gels and contained no aspecific nicking activity. *Micrococcus luteus* UV endonuclease was purchased from Applied Genetics Inc.

#### 2. Plasmid DNA

Plasmid pIC283 is a derivative of pIC20R (14) and contains part of a human cDNA insert cloned between BamHI and KpnI in the multiple cloning site. The plasmid has a size of 3100 bp and was purified from *Escherichia coli* strain HB101 by using standard procedures. The theoretical linking difference  $\Delta LK$  (= difference in linking number between a given topoisomer and the same plasmid in a relaxed state) of native pIC283 was calculated by assuming a superhelical density (or specific linking difference)  $\sigma = -0.06$  (15,16,17) and by using the formula:

$$\sigma = \Delta LK / LK^0 \quad \text{where } LK^0 = N / 10.5$$

N is the number of base pairs of the plasmid and 10.5 the number of bases per turn of the double helix (18). The linking difference  $\Delta LK$  defines the number of superhelical turns of a given plasmid molecule. UV irradiation of plasmid DNA was performed with a germicidal UV lamp (predominant wavelength: 254 nm). Small drops of a 100  $\mu\text{g/ml}$  solution of plasmid pIC283 in TE buffer were put on an ice-cooled petri dish and irradiated with a fluence of 2  $\text{J/m}^2$ . Under these conditions approximately 0.07 pyrimidine dimers are produced per second per plasmid molecule. Singly nicked plasmid DNA was obtained after incubation of 20  $\mu\text{g}$

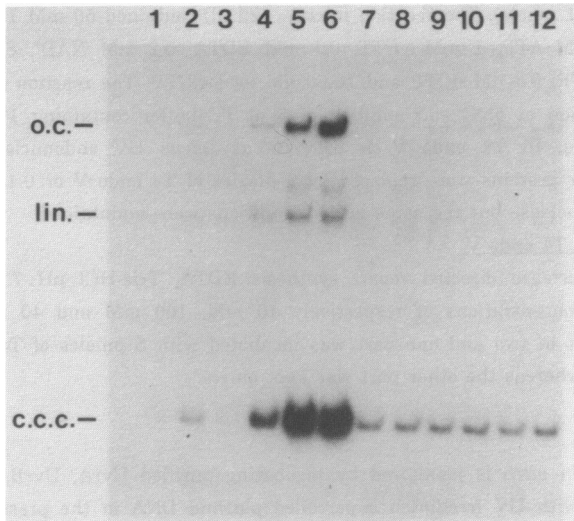


Fig. 1: Autoradiogram of an electrophoretic analysis (gel system I - Materials and Methods) of plasmid pIC283 harbouring either an average of 1 (lanes 1, 4, 7, 10), 2 (lanes 2, 5, 8, 11) or 5 (lanes 3, 6, 9, 12) pyrimidine dimers/molecule incubated under standard conditions with the following proteins: lanes 1-3: UvrA, UvrD, PolA, Lig; lanes 4-6: UvrA, UvrB, UvrC, UvrD, PolA, Lig; lanes 7-9: UvrB, UvrC, UvrD, PolA, Lig; lanes 10-12: PolA, Lig.  
o.c. = open circular (nicked) plasmid; lin = linear plasmid form; c.c.c. = covalently closed circular plasmid DNA.

pIC283 for 1 hr in 1 ml of a buffer containing 20 mM Tris-HCl pH 7.6, 10 mM MgCl<sub>2</sub>, 150 mM NaCl, 100 µg BSA, 300 µg ethidium bromide and 2 µg DNase I of *Escherichia coli*. The reaction was stopped by phenol extraction and the singly nicked plasmid form was purified by electrophoresis on gel system I (see hereafter).

### 3. Electrophoretic techniques.

All gels used were 1.2% agarose gels in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA pH 8.3) and were run in TBE as vertical slab gels (200x200x3 mm) at 40 V for 18 to 21 hr. After electrophoresis gels were dried onto Whatman 3MM paper by using a Biorad Model 224 gel slab dryer. Dried gels were autoradiographed.

\* Gel system I: 1.1 µg/ml of ethidium bromide was included into gel and buffer.

\* Gel system II contained no extra additions.

\* Gel system III: The first dimension was performed following the standard method without extra additions (= gel system II). After completion of the first dimension the gel was removed from the glass plates and soaked for 4 hr in TBE buffer containing 10 µg/ml chloroquine. The gel was then turned 90° and electrophoresed at 40 V for another 18 to 21 hrs in TBE buffer containing 10 µg/ml chloroquine.

### 4. Standard reaction conditions

0.1 µg pIC283 (0.05 pmoles) was incubated for 30 min at 37°C with the following purified proteins: UvrA (3 pmoles), UvrB (3 pmoles), UvrC (2 pmoles), UvrD (2 pmoles), PolA (1

pmole) and Lig (1 pmole). The reaction mixture (25  $\mu$ l) contained 50 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 1 mM DTT, 0.1 mM EDTA, 0.2 mM NAD<sup>+</sup>, 8  $\mu$ M dATP, 8  $\mu$ M dGTP, 8  $\mu$ M dTTP, 0.8  $\mu$ M dCTP and 10-50 nM  $\alpha$ -<sup>32</sup>P-dCTP. The reaction was terminated by a 5 min incubation at 65°C and addition of 5  $\mu$ l TE buffer containing 1% SDS and 0.3 % bromophenol blue. In T4 endo V or *Micrococcus luteus* UV endonuclease driven repair reactions the Uvr proteins were replaced by 5 pmoles of T4 endo V or 0.1  $\mu$ g of *Micrococcus luteus* UV endonuclease but the same buffer conditions were maintained.

### 5. Post-incision with T4 endo V

After Uvr excinuclease directed repair synthesis EDTA, Tris-HCl pH 7.5 and NaCl were added to final concentrations of respectively 10 mM, 100 mM and 40 mM. The reaction mixture was split in two and one part was incubated with 5 pmoles of T4 endonuclease for 30 min at 37°C whereas the other part was kept on ice.

## RESULTS

Excision repair *in vitro* is monitored by incubating purified UvrA, UvrB, UvrC, UvrD, Lig and PolA proteins with UV irradiated supercoiled plasmid DNA in the presence of <sup>32</sup>P labeled deoxynucleotides under the standard reaction conditions described in Materials and Methods. Labeled reaction products are analysed by autoradiography after electrophoretic separation in specific agarose gels. If the complete repair system is used (fig.1: lanes 4, 5, 6) the majority of the label is incorporated into the covalently closed plasmid form and only minor amounts are found in the nicked molecules. The labeling of linear DNA is very weak. Increasing the amount of DNA damage in the plasmid molecule resulted in increased incorporation of labeled nucleotides which can be expected if plasmid labeling is a direct result of repair synthesis. This is further substantiated by the following observation: DNA damage-dependent incorporation of radioactivity into covalently closed plasmid DNA is no more observed if either UvrA is omitted (lanes 7-9) or UvrB and UvrC (lanes 1-3) are left out during the incubation (the same result is obtained when the UvrB or UvrC components are omitted individually - not shown). The consistent low level incorporation of radioactivity into c.c.c. DNA in these control experiments is due to nick-translation initiated on nicked plasmid molecules present at a low percentage in the original plasmid DNA preparation. The same labeling is observed when plasmid DNA is incubated solely with PolA and Lig (lanes 10-12) and is not influenced by the amount of DNA damage (compare lanes 10-12 to lanes 4-6). We have controlled that the PolA and Lig preparations did not contain any contaminating nicking activity (not shown).

In order to be able to specifically follow the repair reaction it was important to find a more accurate way to discriminate between repair synthesis and nick-translation, rather than relying on mere quantitative differences. In figure 2 the reaction products obtained after repair synthesis are analysed on gel system II (Materials and Methods) and compared to the products obtained in a control reaction where the UvrA component has been omitted. The covalently closed circular molecules obtained after repair synthesis behave now as a large number of different topoisomers, extending from the position of the original supercoiled DNA (s.c.) to the position of the open circular (o.c.) plasmid form (Fig. 2: lanes 1-3). In the case of nick-translation a more restricted number of plasmid isomers is observed (lanes 4-6). With increasing amounts of

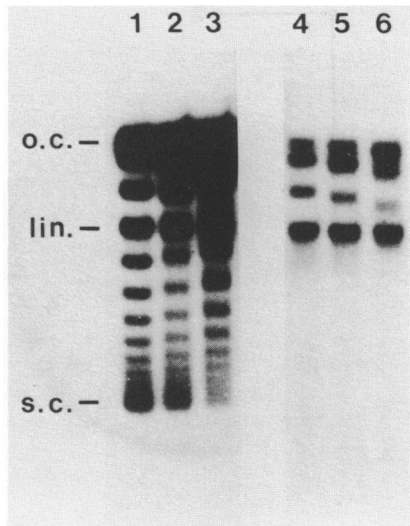
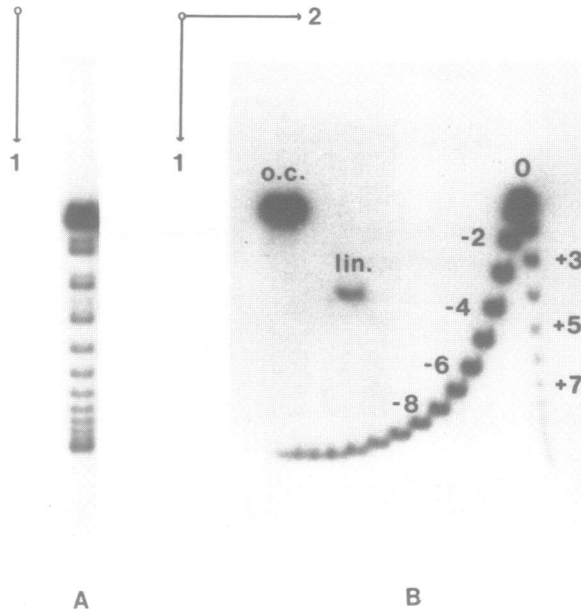


Fig. 2: Autoradiogram of a 1-D analysis (gel system II) of reaction products obtained after excision repair with UvrABCD, Pol A and Lig under standard conditions (lanes 1-3) or under identical conditions but the UvrA component was omitted (lanes 4-6). Plasmids contained an average of either 2 (lane 1 and 4), 5 (lane 2 and 5) or 10 (lane 3 and 6) pyrimidine dimers/molecule. o.c. and lin. are defined in the legend of figure 1. sc = position of the native supercoiled plasmid.

DNA damage in the original plasmids, topoisomers obtained after UvrABC mediated excision repair show reduced mobility whereas topoisomers produced during nick-translation migrate faster in the gel system of figure 2. As UV induced DNA damage (mainly pyrimidine dimers) decreases the number of negative superhelical turns in covalently closed circular plasmid molecules (19) this pattern suggests that radioactive topoisomers visible after DNA repair synthesis are negatively supercoiled whereas nick-translation produces plasmid molecules with a positive superhelical structure. This has been confirmed by 2-D gel analysis (7,15): after repair synthesis topoisomers with both a negative and a positive superhelical structure are produced although the vast majority of these labeled molecules is negatively supercoiled (fig. 3). The ligated radioactive plasmid molecules obtained after nick-translation are on the contrary confined to positively supercoiled topoisomers (fig. 4), which is in accordance with similar observations by others (20). Hence, molecules with a positive linking difference  $\Delta LK$ , obtained during the repair reaction are at least in part the result of nick-translation. The appearance of labeled topoisomers with a negative  $\Delta LK$ , however, seems to be solely the result of excision repair, initiated by the coordinated action of the UvrA, UvrB and UvrC gene products. Indeed if one of these components (UvrA) is omitted during the reaction the negatively supercoiled plasmid forms are no more observed (Fig. 2: lanes 4-6). A similar result is obtained if either UvrB or UvrC is left out (not shown).

During the repair process partial relaxation of the original negatively supercoiled plasmid



**Fig. 3:** Autoradiogram of a two-dimensional electrophoretogram (panel B) (gel system III) of the products obtained after UvrABC mediated excision repair by using the standard conditions described in Materials and Methods. The distribution of topoisomers after electrophoresis in the first dimension is shown in panel A. The numbers indicate the value of the linking difference  $\Delta LK$  of a given topoisomer. o.c. = open circular form; lin = linear plasmid form. (For discussions on the interpretation of the two-dimensional electrophoretograms refer to (15).

DNA is observed (figs.2 and 3). Native plasmid substrates isolated from the *Escherichia coli* strain HB101 were in general composed of 7 major topoisomers (not shown) with an average calculated  $\Delta LK$  of -18 (Materials and Methods). After repair synthesis on the contrary a whole range of different topoisomers is produced spanning from topoisomers with  $\Delta LK = -16$  (which is the limit of resolution of the gel system used) to completely relaxed double-stranded circles ( $\Delta LK = 0$ ). The appearance of all these topological forms suggests that after incision of the damaged plasmid by the Uvr excinuclease the superhelical stress in the molecule is at least partially maintained; indeed we have shown (Fig. 4) that resealing of completely relaxed (nicked) plasmids results in topoisomers with positive supercoiling. Above all it is interesting to point out that after excision repair labeled DNA molecules are encountered, which have maintained a very substantial amount of supercoiling (more than 12 negative superhelical turns). It should be clear that during the repair of these molecules the Uvr proteins were able to carry out incision, displacement of the damage containing oligonucleotide, repolymerization of the gap and ligation without greatly relaxing the DNA template (in a 1-D analysis these topoisomers migrate at the same position as native plasmids: s.c. in Fig. 2). These results have prompted us to analyse whether this ability is specific for the Uvr-triggered repair system. Furthermore we were interested in studying the mechanism by which such highly supercoiled products are formed.

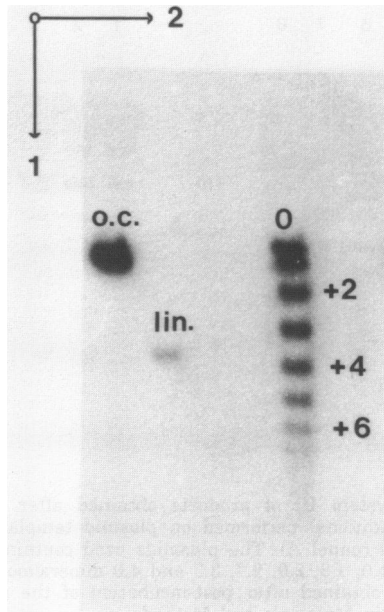


Fig. 4: 2-D analysis of singly nicked plasmid pIC283 (see Materials and Methods) incubated with PolA and Lig under standard reaction conditions. Annotations are as in figure 3.

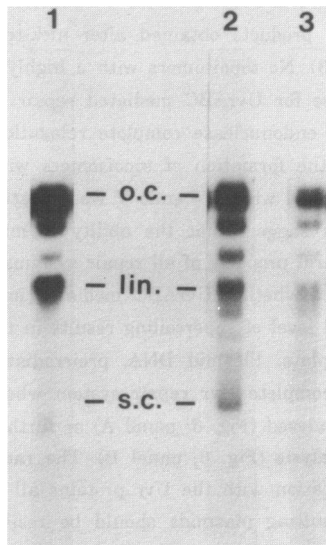
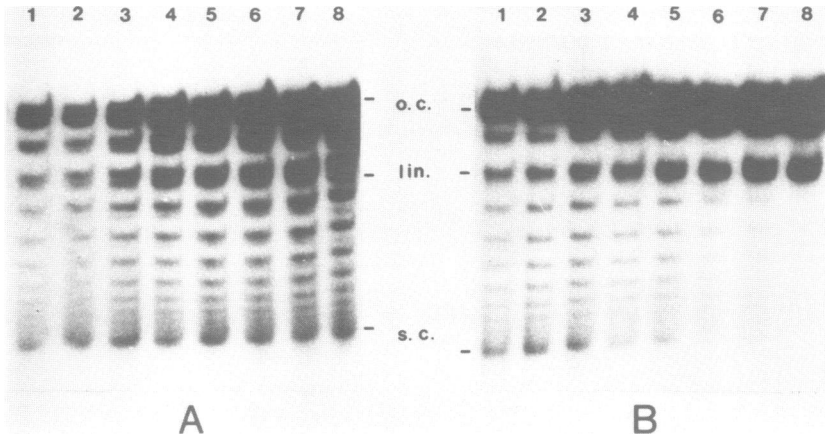


Fig. 5: Gel system II was used to compare radioactive products obtained when plasmid pIC283 (4 pyrimidine dimers/molecule) was incubated under standard conditions with the following proteins: lane 1: T4 endo V, UvrD, PolA, Lig; lane 2: UvrA, UvrB, UvrC, UvrD, PolA and Lig; lane 3: identical to lane 2 but UvrA was omitted. Annotations are as in figure 2.



**Fig. 6:** 1-D analysis (gel system II) of products obtained after UvrABC dependent excision repair (standard conditions) performed on plasmid templates with increasing amounts of pyrimidine dimers (panel A). The plasmids used contained from lane 1 to lane 8 an average of 0.3; 0.7; 1.0; 1.3; 2.0; 2.7; 3.3 and 4.0 dimers/molecules respectively. Panel B shows the products obtained after post-incubation of the samples in panel A with T4 endo V as described in Materials and Methods.

In figure 5 we have compared UvrABC mediated repair to the repair synthesis induced by T4 endonuclease V. The labeled products obtained after T4 endonuclease mediated repair synthesis are qualitatively similar to the products obtained after nick-translation of the same plasmid template (compare lanes 1 and 3). No topoisomers with a highly negative superhelical structure (s.c.) are obtained as is the case for UvrABC mediated repair (lane 2). The analysis suggests that during incision by the T4 endonuclease complete relaxation of the plasmid DNA occurs, which after ligation results in the formation of topoisomers with a positive linking difference  $\Delta LK$ . Identical results were obtained when T4 endo V was substituted by the *Micrococcus luteus* UV endonuclease. These results suggest that the ability to maintain substantial supercoiling during DNA repair is not a general property of all repair systems.

In figure 6 we have controlled whether UvrABC mediated incorporation of radioactivity into topoisomers with a high residual level of supercoiling results in the actual removal of pyrimidine dimers from the damaged template. Plasmid DNA, preirradiated with different doses of UV light, was incubated with the complete Uvr repair system whereafter the radioactive plasmid products were either directly analysed (Fig. 6, panel A) or further incubated in the presence of T4 endo V prior to 1-D gel analysis (Fig. 6, panel B). The rationale of this experiment is as follows: If during the first incubation with the Uvr proteins all pyrimidine dimers are removed during repair synthesis the resulting plasmids should be resistant to the attack by the T4 enzyme. If, however, not all the dimers are removed from a given plasmid than incision by the T4 enzyme will occur resulting in the appearance of nicked (o.c.) or linear (lin) molecules. The analysis in figure 6 indicates that up to a UV dose of  $40 \text{ J/m}^2$  the highly supercoiled plasmid



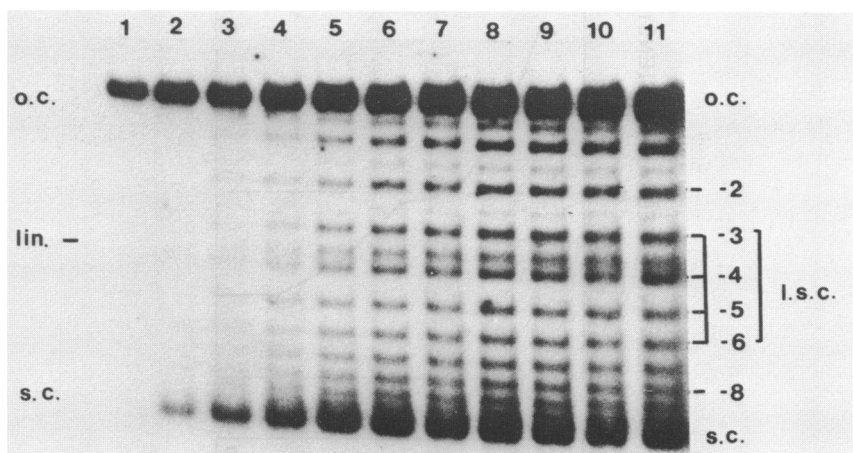


Fig. 7: Autoradiogram of a 1-D analysis of excision repair products obtained after different times of incubation of plasmid pIC283 (average of 1 dimer/molecule) with the complete standard reaction mixture. From lane 1 till lane 11 the incubation time was: 2.5; 5; 7.5; 10; 15; 20; 25; 30; 40; 50; and 60 min respectively. -2 and -8 indicate the position of topoisomers with  $\Delta LK = -2$  and  $\Delta LK = -8$  respectively. l.s.c. indicates the position of topoisomers with low supercoiling ( $\Delta LK = -3$  to  $-6$ ) used in the calculation in fig. 8A.

forms obtained after UvrABC mediated repair synthesis are free of pyrimidine dimers. The amount of label at the s.c. position in lanes A1 to A3 is not significantly different from the one in lanes B1 to B3. If, however, plasmid DNA is irradiated with higher doses of UV (lanes 4-8) not all dimers are being repaired by the Uvr system which results in plasmid relaxation after T4 endo V incubation (panel B). From these data we can deduce that although dimer excision occurs the complete removal of all dimers from one plasmid molecule is rather inefficient as our Uvr excinuclease preparation is not able to remove more than one dimer per plasmid without inducing gross changes in the superhelical plasmid structure. Indeed, increasing the amount of DNA damage per plasmid results in a gradual relaxation of the labeled plasmid molecules during repair synthesis (Fig. 2 and fig. 6). In figure 2 inefficient repair is visible by the different mobility of topoisomers due to unremoved pyrimidine dimers.

In figure 7 a kinetic analysis has been performed in order to follow the appearance of repair label in the different topoisomeric forms in time. From this analysis a number of conclusions can be drawn:

The incorporation of label into the open circular (o.c.) form is very rapid and reaches a plateau after 5 min. of incubation<sup>a)</sup>. This labeling is due to nick-translation and probably initiated before incubation of the reaction mixture at 37°C (see figure 8B) as nick-translation can occur at low temperature (21).

<sup>a)</sup> On the photograph the labeling of open circular molecules seems to increase during the whole reaction; on the original autoradiogram, however, it is clearly visible that this increase is due to the gradual appearance of topoisomers with  $\Delta LK=0$  which migrate just above o.c. (fig. 3)

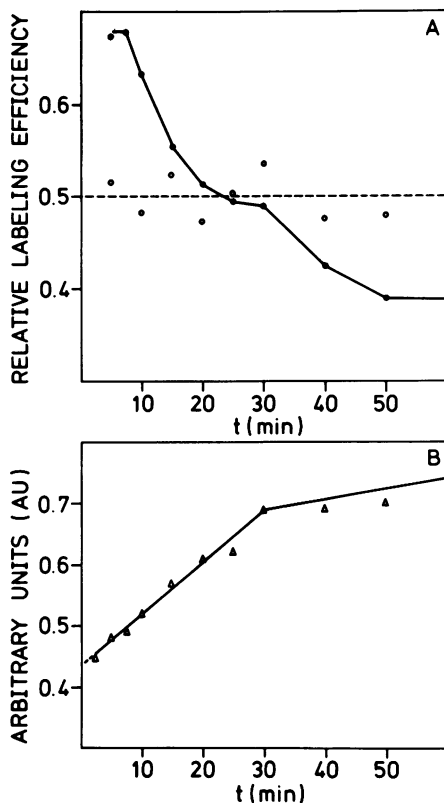


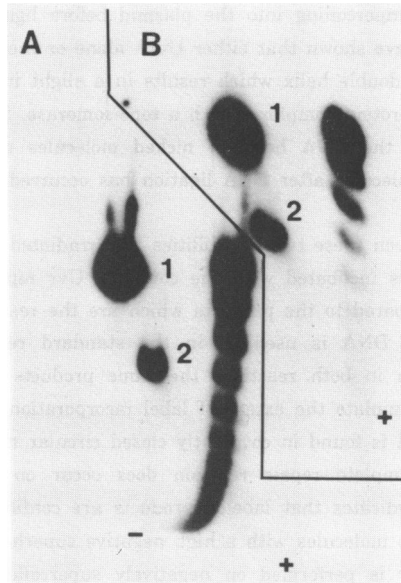
Fig. 8: A) Relative labeling efficiency of highly supercoiled plasmid forms ( $\Delta LK = -13$  to  $-16$ ) versus plasmids with lower supercoiling ( $\Delta LK = -3$  to  $-6$ ) (closed symbols) and topoisomers with  $\Delta LK = -8$  versus topoisomers with  $\Delta LK = -2$  (open symbols). The relative labeling efficiency of highly supercoiled plasmids or topoisomers of  $\Delta LK = -8$

is defined as the ratio:  $\frac{[s.c.]}{[s.c.] + [l.s.c.]}$  or  $\frac{[-8]}{[-8] + [-2]}$  respectively where [x] represents

the amount of label incorporated into the indicated topological plasmid form or population as determined after scanning the autoradiogram of fig. 7 with an LKB Ultrascan laserscanner. The stippled line represents the theoretical curve expected if two (populations of) topoisomers are labeled with identical kinetics; s.c. and l.s.c. are defined in the text.

B) Kinetics of incorporation of total repair label expressed in arbitrary units (AU) obtained after scanning of the different lanes in figure 7 with the LKB laserscanner.

At initial stages of the reaction the incorporation of label into circular covalently closed molecules is mainly confined to plasmid forms with a high superhelical density migrating at the position of the original supercoiled template (s.c.). The labeling of these molecules which have a  $\Delta LK$  of  $-13$  to  $-16$  (see fig. 3) increases up to 10 min whereafter a plateau is reached. From that moment on a more extensive labeling of plasmids with a lower superhelical density is observed (fig. 7).



**Fig. 9:** 2-D electrophoretograms of the products obtained after UvrABC directed excision repair performed under standard conditions on either supercoiled plasmid (pattern A) or singly nicked plasmid (pattern B) and resolved on the same gel (second dimension run for 10 hours only). Both plasmid preparations contained an average of 1 dimer/molecule. A1 and A2 represent the o.c. and linear forms belonging to pattern A; B1 and B2: the same for pattern B.

In figure 8A we have directly compared the appearance of label in the population of plasmids having a  $\Delta\text{LK}$  from -13 to -16 (s.c.) to a population of plasmids (l.s.c.) with 10 helical turns less ( $\Delta\text{LK}$  -3 to -6) (closed symbols) as well as the labeling of a topoisomer with  $\Delta\text{LK} = -8$  to a topoisomer with  $\Delta\text{LK} = -2$  (open symbols). At initial stages of the reaction the majority of the label is incorporated into highly supercoiled molecules whereas at late stages the labeling of more relaxed plasmid forms dominates (fig. 8A, closed symbols). This apparent change in specificity is paralleled by a decrease in the rate of total label incorporation (fig. 8B). On the contrary, the labeling of topoisomers with  $\Delta\text{LK} = -8$  and  $\Delta\text{LK} = -2$  occurs with similar kinetics (relative labeling efficiency = 0.5) during the entire course of the reaction (fig. 8A, open symbols). This finding suggests that the observed relaxation is probably not the result of an active mechanism inherent to the Uvr excinuclease (see discussion).

Two different mechanisms could account for the presence of supercoiled DNA molecules after repair synthesis:

1. The UvrA, B and C proteins have the ability to restrain the superhelical stress present in the template and incision, repair synthesis and ligation occur in a concerted manner. The Uvr excinuclease (or one of its components) is only released after ligation is completed.
2. Incision of supercoiled template DNA by the Uvr excinuclease results in complete release of the original superhelical stress of the molecule. The Uvr proteins, however, have the ability

to reintroduce negative supercoiling into the plasmid before ligation is completed. Recently Oh and Grossman (22) have shown that either UvrA alone or the UvrAB complex are able to locally unwind the DNA double helix which results in a slight increase of the negative  $\Delta LK$  after treating the nucleoprotein complexes with a topoisomerase. Similarly one could envisage that local unwinding of the DNA helix in nicked molecules results in the formation of negatively supercoiled molecules after DNA ligation has occurred and the Uvr proteins have been removed.

In order to distinguish between these two possibilities UV irradiated singly nicked plasmid DNA (Materials and Methods) was incubated with the complete Uvr repair system and the labeled products obtained were compared to the products which are the result of the standard reaction where supercoiled template DNA is used. If in the standard reaction relaxed DNA is an obligatory intermediate then in both reactions the same products should be observed. When nicked DNA is used as a template the extent of label incorporation increases linearly with the amount of DNA damage and is found in covalently closed circular molecules after DNA ligation (not shown). Hence the complete repair reaction does occur on a relaxed DNA template. Nevertheless our analysis indicates that labeled products are confined to positively supercoiled topoisomers (Fig. 9B) and no molecules with a high negative superhelical structure are observed as is the case when repair is performed on negatively supercoiled plasmids (Fig. 9A). This experiment strongly suggests that relaxed DNA is not an obligatory intermediate during repair synthesis promoted by the Uvr excinuclease. Apparently, the Uvr repair system has the ability to maintain the superhelical stress present in the template DNA during repair synthesis.

### DISCUSSION

In this communication we have used an *in vitro* system containing purified UvrA, UvrB, UvrC, UvrD, PolA and Lig as well as UV irradiated plasmid DNA in order to study the effect of nucleotide excision repair on the superhelical structure of DNA. The method consists of analysing labeled plasmid molecules obtained after DNA repair on specific gel systems which allow assessment of their superhelical conformation.

Such an approach was possible since we were able to distinguish between reaction products which are the result of nucleotide excision repair or nick-translation of the DNA template. The use of this radioactive approach is necessary in order to visualize and quantify the plasmid molecules which have undergone excision repair. Although the different topoisomeric forms obtained after incubation with the repair enzymes can be visualized after electrophoresis by ethidium bromide staining an accurate measurement of the fraction of highly supercoiled molecules which have undergone reaction is not possible in this way, especially if short reaction times and plasmid DNA with a low dimer density are used, conditions where repair with restraint of superhelicity was found to be the most efficient. Under such conditions repaired supercoiled molecules are masked by an excess of molecules which have not undergone reaction. One possible disadvantage of using the radioactive method for quantification purposes is that we have to assume that the repair patches produced in each of these topoisomers have approximately the same size. Comparison of the intensity of the different topoisomeric forms with lower supercoiling (1 to 8 negative superhelical turns) in ethidium stained gels to the

corresponding autoradiogram suggests that in this case our assumption seems justified. At present we cannot completely be sure that this is also true if we compare highly supercoiled topoisomers ( $\Delta LK > -12$ ) with low supercoiled molecules. However this does not invalidate our observation that repaired molecules with a residual high level of supercoiling are produced and might only affect the accuracy with which such products can be quantified.

Labeled reaction products with high negative supercoiling were only detected when the repair reaction was performed on a negatively supercoiled template DNA but not if relaxed plasmids were used. This result suggests that the UvrABC directed repair system is able to restrain the superhelical tension in a DNA molecule during the repair process rather than introducing, by means of a gyrase-like activity, new negative superhelicity into a molecule which has been completely relaxed after the incision step. This ability to restrain superhelicity, observed in the case of DNA repair initiated by the coordinate action of UvrA, B and C, was not found when the repair reaction was performed with T4 endonuclease V. Apparently the functions necessary for this specific activity are carried by the UvrA, B and C proteins and not by the UvrD, PolA and LigA components which were also present in the T4 endo V reaction.

The efficiency by which our Uvr excinuclease preparation is able to restrain superhelical stress during excision repair in our *in vitro* system is rather weak. The analysis shows that in the course of the reaction topoisomers with a lower superhelical density are readily generated. A kinetic analysis suggests that the formation of these topoisomeric forms with low superhelicity is not due to an inherent property of the Uvr system. As a matter of fact, if topoisomers with a low superhelical density were generated by the Uvr proteins by a controlled mechanism (c.f. class I topoisomerases), one should expect that the different products would appear gradually in time with more relaxed forms present at later stages of the reaction. Our analysis clearly shows that topoisomers with  $\Delta LK = -2$  and  $\Delta LK = -8$  are generated with similar kinetics during the entire incubation period by an apparently completely random process. At present we cannot be completely sure about what the molecular reason for this apparent inefficiency of our *in vitro* system might be. It is interesting in this respect to mention that recently Gruskin and Lloyd (25) have observed that *in vivo* nucleotide excision repair mediated by UvrABC can be completed without any detectable relaxation of the DNA template. When the same *in vivo* system was used to monitor excision repair mediated by T4 endonuclease V the accumulation of nicked plasmid intermediates was readily observed (24). Inefficient restraint of superhelicity *in vitro* could have several reasons:

a) Inefficiency could be due to partial inactivation of the purified proteins which results in a rapid decrease in the ability of the Uvr excinuclease to restrain superhelical stress during the course of the reaction. It should be clear that the function responsible for restraining negative supercoiling in DNA can only be partially inactivated as complete relaxation of the plasmid template would result in the generation of topoisomers with a positive linking difference after ligation as found when incision is performed with T4 endonuclease V or *Micrococcus luteus* UV endonuclease. That partial inactivation of the purified proteins can readily occur is evident from the following observation: When Uvr proteins (especially the UvrB and UvrC subunits) were diluted in reaction buffer at the standard concentrations and left on ice for 30 to 60 min before the start of the reaction the ability to incorporate label into highly supercoiled plasmid forms

was strongly diminished, although incision of damaged DNA did still occur. Apparently the functions involved in restraining superhelicity are very prone to inactivation. At present we have no molecular explanation for this observation. Recently, Selby and Sancar (23) observed that "aged" preparations of purified Uvr proteins (2 weeks at  $-20^{\circ}\text{C}$ ) show uncoupled incision restricted to the 3' site in 80% of the cases. It is conceivable that double incision is a prerequisite for repair in supercoiled templates and that uncoupled incision is the reason for the partial inactivation and the rapid relaxation which we observe. More experiments are needed to clarify this point.

b) Inefficiency of the Uvr excinuclease to restrain supercoiling *in vitro* could be due to a difference in superhelicity between purified and cellular plasmid DNA. We have mentioned earlier that stress induced by DNA supercoiling is partially stabilized by nucleosome-like structures *in vivo*. It has been shown that the superhelical density of intracellular DNA can be estimated to be around -0.03 to -0.04 as compared to a value of  $\sigma = -0.06$  for the same DNA after purification (9). Consequently the torsional tension in purified DNA might be slightly too high in order to be efficiently restrained by the Uvr repair system.

c) Finally, it is not impossible that we miss an enhancing protein factor in our *in vitro* system. It will be interesting to analyse the effect of different DNA binding proteins in our test system.

At present our analysis does not allow to decide on which of the 3 excinuclease subunits the domains involved in the restraining of torsional tension are localized. Our inactivation data (mentioned above) suggest that both UvrB and UvrC might be involved, which is in accordance with the recent model proposed by Sancar's group (26), where UvrA leaves the specific complex before incision occurs. However the isolation of specific mutants in the three genes will be necessary to clarify this point.

The ability of the Uvr proteins to restrain DNA superhelicity during the repair process is shared by other enzymatic processes (e.g. integrases, transposases, resolvases and invertases) which incise the DNA backbone (27). The experiments described here do not allow to determine whether nucleotide excision repair by the Uvr system results in a slight change of the DNA superstructure as has been found for several of the systems mentioned above. For instance in the case of Gin-mediated DNA inversion (double incision on both DNA strands) completion of the reaction is accompanied with the loss of 4 superhelical turns (28). Such a variation in superhelicity constitutes a topological signature of a given enzyme and reveals the basic features of its mechanism. In the case of the Gin invertase this topological method has allowed to predict the structure of the synaptic complex which could then be further substantiated later on (29). In order to apply the topological method to the Uvr excinuclease it will be necessary to use plasmid templates with a single DNA adduct and a fixed number of superhelical turns.

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