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PERTURBATION OF DNA TERTIARY STRUCTURE BY PHYSICAL AND CHEMICAL CARCINOGENS: EFFECTS ON DNA REPAIR PROCESSES

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Summary. - *DNA within the cell is organized into higher-order structures characterized by negative supercoiling. Supercoiling is a property of any DNA molecule lacking ends capable of rotation. Parameters defining the properties of supercoiled DNA are significant for the description of the reactive state of DNA molecules. We have investigated whether physical and chemical DNA modifying agents alter the parameters describing the DNA tertiary structure. The variations in DNA tertiary structure of partially relaxed topoisomers obtained from plasmid DNA have been studied by one dimensional agarose gel electrophoresis, a technique allowing the measurement of alterations in the degree of supercoiling equivalent to fractions of superhelical turns. Unwinding angles of 8.5° for pyrimidine dimers and of 8.5° for acetyl-4-hydroxyaminoquinoline-1-oxide (Ac-4-HAQO) adducts have been determined by titrating for each topoisomers the number of damaged sites necessary to reduce the superhelical turns by one. Analogous unwinding was observed for topoisomers obtained from* in vivo *irradiated plasmid DNA. We have also shown that local alterations in DNA structure caused by UV irradiation inhibit bacterial type I DNA topoisomerases. In addition, we have demonstrated that* E. coli *mutants lacking DNA topoisomerase I are sensitive to UV light. The pronounced inhibition of DNA synthesis as well as the chromosome instability observed after UV irradiation of this strain, suggest that DNA topoisomerase I might be involved in those cellular responses elicited by the proximity of damaged bases to sites of active replication. These findings indicate that the DNA structural alterations deriving from DNA damage formation may influence DNA repair processes either by driving the interaction between repair enzymes and modified sites on DNA, or by inhibiting enzymes normally acting on unmodified DNA.*

Riassunto (Perturbazioni della struttura terziaria del DNA causate da carcinogeni chimici e fisici: effetti sui processi riparativi). - *Il DNA nella cellula è organizzato in strutture di ordine superiore caratterizzate dall'essere negativamente superavvolte. Il superavvolgimento è una proprietà che caratterizza ogni molecola di DNA mancante di estremità capaci di ruotare liberamente. I parametri che definiscono le proprietà del DNA superavvolto sono importanti nel descrivere la reattività delle molecole di DNA. Noi abbiamo indagato sulla possibilità che agenti chimico fisici, che danneggiano le basi, alterino la struttura terziaria del DNA. Queste alterazioni sono state studiate mediante elettroforesi monodimensionale su gel di agarosio di topoisomeri ottenuti dal rilassamento di DNA plasmidico. Questa tecnica consente di misurare variazioni nel grado di superavvolgimento equivalenti a frazioni di giro di superelica (360°). Titolando il numero di siti danneggiati necessari a ridurre il superavvolgimento di ciascun topoisomero di una unità, abbiamo calcolato le variazioni nell'angolo di avvolgimento della doppia elica causate dai dimeri di pirimidina e dagli addotti dell' acetil-4-idrossiaminochinolina-1-ossido (Ac-4-HAQO). Una variazione analoga è stata misurata anche in topoisomeri ottenuti da DNA plasmidico irraggiato* in vivo. *Abbiamo inoltre osservato che le alterazioni locali della doppia elica causate dai raggi ultravioletti inibiscono le DNA topoisomerasi batteriche di tipo I. Da ultimo abbiamo mostrato che mutanti di* E. coli *mancanti della DNA topoisomerasi I sono sensibili alla luce ultravioletta. Il trattamento di questi mutanti con UV a 254 nm ha causato una inibizione marcata della sintesi del DNA e una instabilità del cromosoma, indicando che la DNA topoisomerasi I potrebbe essere coinvolta nei processi riparativi. Queste osservazioni suggeriscono che le alterazioni della struttura del DNA prodotte in seguito*

alla formazione di danni possono influenzare la risposta cellulare sia guidando l'interazione tra gli enzimi della riparazione e i siti danneggiati, sia inibendo gli enzimi che normalmente agiscono sul DNA.

Introduction

Damaging agents induce cytotoxic, mutagenic, and carcinogenic changes in the cells. All these biological changes are considered to be the consequence of modifications to DNA. Therefore, a detailed knowledge of the chemical and structural alterations that occur in DNA is important for the understanding of the biological effects of DNA damage, as well as for the role that these modifications play in the enzymology of recognition and repair of specific DNA lesions [1].

Several evidences have indicated that interaction between DNA and damaging agents has effects on the structure and stability of the DNA helix, and it has been suggested that such effects may be significant with respect to modulation of DNA-protein interactions. In fact, alterations in conformation at the glycosylic bond, rotation of the backbone residues, and sugar puckering consequent to damage formation, are expected to act as recognition signals for DNA repair enzymes as well as to interfere with the normal reaction of proteins involved in DNA metabolism [2].

DNA within the cell is not a simple, rigid double helix but it is a flexible molecule organized into complex higher order structures characterized by negative supercoiling, an ubiquitous property of DNA extracted from natural sources [3]. A very important point about negative supercoiling is that supercoiled DNA possesses a greater free energy than relaxed DNA. Thus, supercoiled DNA can be considered in an active state facilitating all reactions which tend to relax DNA, such as B-Z transition, hairpin formation, sequence specific binding of proteins, binding of DNA ligands [4]. Although the role of supercoiling in eukaryotes is still obscure, it is clear that in prokaryotes it plays an important role in transcription, replication, and recombination [5]. Less clear is instead the relationship between supercoiling and DNA repair, although indications of a possible involvement in cell recovery from UV damage come from the UV sensitivity of mutants altered in genes coding for proteins responsible for chromosome superhelicity [6].

To assess the effects of DNA damage on DNA tertiary structure as well as on the interaction between proteins and damaged DNA, we have studied: a) alterations of DNA conformation on *in vitro* UV-irradiated, depurinated and chemically modified circular closed DNA molecules; b) effect of damage induced alteration upon enzymes controlling the topological state of DNA; c) effect of mutations in genes responsible for chromosome supercoiling on DNA repair processes.

Structural perturbations of DNA helix caused by carcinogens

Alterations in the native helical conformation associated with the formation of DNA damage have been studied by using various forms of spectroscopy, electric linear and circular dichroism, velocity sedimentation, electron microscopy, sensitivity to single-strand specific endonucleases and reagents, and by using oligonucleotides or linear polymeric nucleic acids as substrates. Recently a more sensitive method has become available, which allows the detection of structural variations caused by lesions present in DNA with a frequency of 10^{-3} to 10^{-4} per base pair. This method, called band shift method, is based on the known topological properties of closed circular DNA molecules which are particularly sensitive to small conformational changes in their tertiary structure [7]. Moreover, because of their superhelical nature, these molecules better mimic the form of intracellular DNA.

Principle of the method

In closed circular DNA the reciprocal relationship between the twisting of the double helix and the supertwisting of the helix axis can be expressed in a quantitative form by the following equation:

$$Lk = Tw + Wr$$

The *linking number* (Lk) is the parameter that describes quantitatively the linking of two complementary single-strand rings, thus it measures the number of times one strand goes about the other. It is an integer and can vary only by breakage and subsequent rejoining of the phosphodiester bonds. The *writhing number* (Wr) defines the path followed by the axis of the double helix (supercoiling is the intuitive expression for Wr). The *twist* (Tw) measures the frequency of periodicity of duplex winding about the helix axis in its native conformation. It depends on the angular displacement (twist angle) between adjacent bases, that in its turn has been shown to depend on temperature, counter-ion concentration, hydration, presence of DNA ligands [7].

Therefore, in a closed DNA molecule, according to the environmental conditions, the Tw and the Wr may fluctuate, but because of the topological constraint their sum must remain constant. DNA molecules differing only in their topological parameter (Lk) are called topoisomers. It has been shown that topoisomers which differ in their linking number by one integral value can be resolved, in appropriate conditions, by agarose gel electrophoresis into a series of sharp bands (Fig. 1). This finding has been interpreted as due to a faster fluctuation of the Wr compared to the electrophoresis time scale. Consequently the electrophoretic mobility of the topoisomers has to be determined by the writhing number.

The covalent modification of DNA bases by damaging agents modulates the electronic distribution of the bases resulting in a change in orientation of the phosphodiester backbone, and thereby in a change in

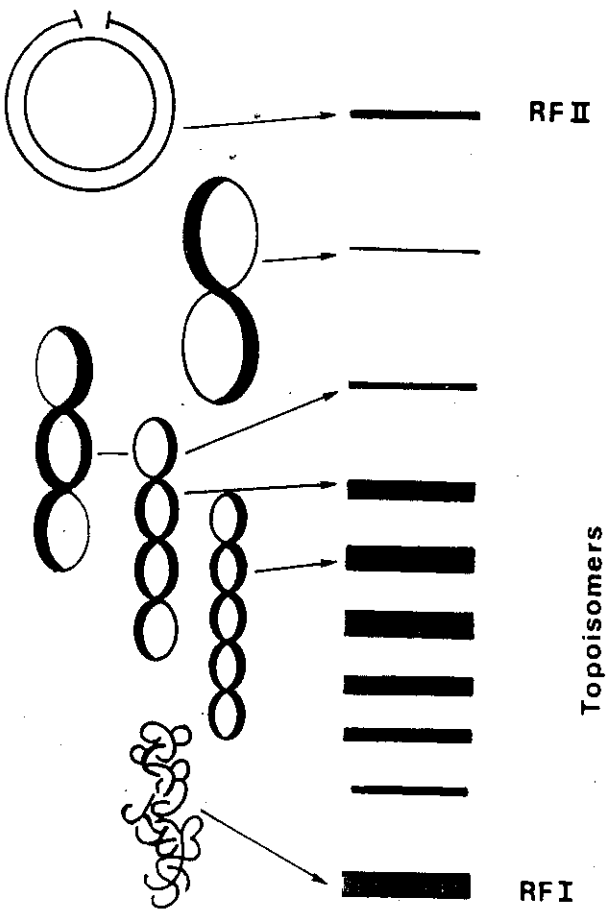

Fig. 1. - Electrophoretic mobility on agarose gel of circular closed supercoiled (RF I), nicked circular (RF II), and partially relaxed topoisomers of pAT153 DNA.

twist. It is evident that in a closed circular DNA molecule, because of the topological constraint, any change in twist must be compensated by a variation in writhing number of equal magnitude and opposite sign.

This is apparent upon consideration of the relationship:

$$Lk_u = Tw_u + Wr_u \text{ and } Lk_d = Tw_d + Wr_d$$

where u and d subscripts indicate undamaged and damaged molecules, respectively. Since for any given topoisomer

$$Lk_u = Lk_d$$

we will also have that

$$Tw_u + Wr_u = Tw_d + Wr_d$$

The difference in helical repeat between the undamaged and damaged DNA will be equal to:

$$Tw_u - Tw_d = Wr_d - Wr_u$$

Thus, a change in twist due to the damage will result in a change in writhe and consequently in the mobility of the topoisomer bands, the magnitude of which is proportional to the difference in the helical repeat of the damaged and undamaged form under the electrophoresis conditions.

The situation changes and becomes more complex when damage is introduced into an open DNA molecule which will be subsequently closed with DNA ligase. At the instant of closure the topological relationships are:

$$Lk_u^* = Tw_u^* + Wr_u^* \text{ and } Lk_d^* = Tw_d^* + Wr_d^*$$

where the superscript indicates the condition of ligation. In this situation Lk_u is not exactly equal to Lk_d. However since Lk can only be an integer, the linking numbers of the undamaged and damaged molecules are related by the expression

$$Lk_d^* = Lk_u^* + I$$

where I is an integer. Thus,

$$Wr_d^* - Wr_u^* = I - (Tw_d^* - Tw_u^*)$$

Since, as previously reported, the geometrical parameters depend on a given set of conditions, the transfer from ligation to gel electrophoresis determines a change in Tw and Wr. But since the linking number cannot change,

$$Tw_u + Wr_u = Tw_u^* + Wr_u^* \text{ and } Tw_d + Wr_d = Tw_d^* + Wr_d^*$$

where the absence of the prime superscript indicates conditions of electrophoresis. Combining these equations one can obtain the following relationship:

$$Wr_d - Wr_u = I + Tw_u - Tw_d$$

Therefore any change in mobility of the topoisomer bands reflects the difference in twist under conditions of electrophoresis plus any damage dependent difference in linking number due to damage locked at the time of ligation. If no difference in linking number is introduced, I is 0, and the mobility changes observed appear the same as those for DNA modified after ligation.

Topoisomers of naturally supertwisted DNA are difficult to resolve electrophoretically due to their high degree of compactness. Thus, to study the effects of small changes in the geometrical parameters of the double helix, it is appropriate to use enzymatically relaxed forms. Partially relaxed topoisomers offer the additional advantage that the Wr is too small to generate constraints which would alter the twist, thus they are expected to migrate more nearly like the nicked-circular forms owing to changes in electrophoresis voltage or gel composition. In fact, Strauss *et al.* [8] have demonstrated that, contrary to what has been observed for naturally supercoiled molecules, the length dependence of topoisomer mobility does not depend on their superhelicity but corresponds to that of the nicked form. Thus, experiments with nicked circular DNA will be useful in predicting when effects other than changes in twist might become important.

Partially relaxed topoisomers are prepared by type I DNA topoisomerases relaxation of circular closed molecules or by ligase closure of singly-nicked DNA circles. Using appropriate conditions in the preparation of partially relaxed topoisomers, it is possible to obtain topoisomers that under the electrophoresis conditions appear as positively or negatively supercoiled, thus allowing to test whether or not changes in twist angle depend on the number and/or the sense of supercoiling.

Damaged DNA has been shown to be characterized by a reduction in the melting profile of the molecule. Analysis of the change in electrophoretic mobility at different temperatures will also allow to establish whether the observed conformational variations are associated with a thermal transition induced by the damage.

The magnitude of the helical conformational transition can be calculated by measuring the relative mobility (R_f) of each topoisomer band with respect to two undamaged markers added to the modified topoisomers prior to electrophoresis. As shown in Fig. 2, one is used as origin (λ-DNA) and the other as front (plasmid RFI DNA). By titrating the number of modified sites necessary to induce a band shift of one superhelical turn, it is possible to estimate the structural transition caused by one site. The structural modification is expressed in degrees on the assumption that a band shift of one unit corresponds to 360° unwinding.

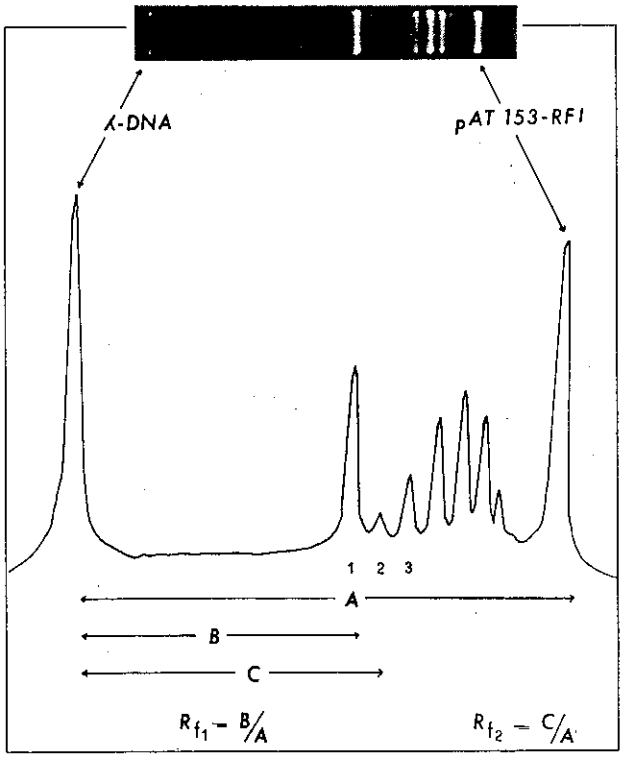

Fig. 2. - Calculation of the relative mobility (R_f) of topoisomer bands respect to two control markers.

Unwinding of the DNA helix induced by physical and chemical damaging agents

When covalently closed relaxed DNA has been irradiated with UV light at 254 nm, depurinated by heat-acid treatment, chemically modified with the ultimate carcinogen of 4-nitroquinoline-1-oxide, the acetyl-4-hydroxyaminoquinoline-1-oxide (Ac-4-HAQO) or with N-acetoxy-2-acetylaminofluorene (N-AcO-AAF), the topoisomer bands observed upon agarose gel electrophoresis are shifted relative to those of the non damaged control molecules. As shown in Fig. 3, in all analyzed cases, we have observed a measurable reduction in electrophoretic mobility of the damaged topoisomers. The reduction is linear with respect to the dose and it is the same for each topoisomer band suggesting that, at least in the range observed, torsional tension does not significantly influence the extent of deformation. On the contrary an influence of superhelical tension on the structural alteration induced by DNA damage has been suggested by studies with benzo(a)pyrene diol epoxide [9] and *cis*-diamminedichloroplatinum(II) [10]. Since topoisomers have been prepared in such a way that they are negatively supercoiled upon gel electrophoresis, the upward shift of each band indicates that damage causes helix unwinding.

Increase in the number of damage per genome causes a progressive loss of resolution of the individual topoisomer bands, effect probably due to the formation of a Gaussian distribution of damage on each topoisomer which in turn produces a continuous set of conformational isomers. Since each conformational isomer only differs by the magnitude of the damage unwinding angle, they are not resolvable by conventional agarose gel electrophoresis.

It is well known that most physical and chemical damaging agents induce a large variety of products. The unwinding angle value per damage is calculated on the assumption that only the most abundant damage formed after a damaging treatment causes helix distortion or that the contribution to the total effect of each damage does not vary between adducts. This assumption has revealed to be incorrect at least in two cases we have analyzed. In

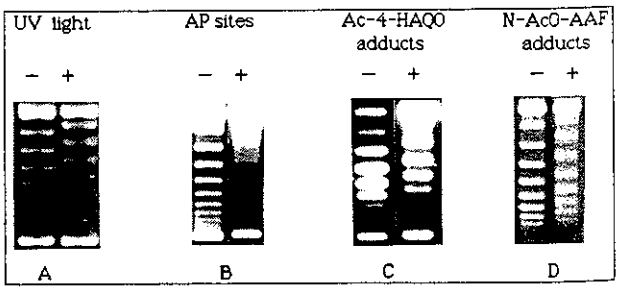

Fig. 3. - Effect of a number of carcinogenic treatments on the electrophoretic mobility of plasmid pAT153 negatively supercoiled topoisomers.

fact, we have observed that complete removal of pyrimidine dimers by photoreactivation does not restore the electrophoretic mobility of the UV irradiated topoisomers at the very level of the undamaged control [11, 12]. In fact, 20% of the original topoisomer mobility shift is not photoreactivable, suggesting that photodamage other than pyrimidine dimers contributes to total unwinding [13]. Moreover in the case of Ac-4-HAQO, we have observed a different unwinding angle per bound carcinogen for positively and negatively supercoiled topoisomers. Since it has been shown that these two forms contain a dissimilar distribution of adducts, the total perturbation we have measured has to originate from a different contribution of the various adducts (unpublished results). In conclusion, it is more appropriate to consider the unwinding angle per damage as representative of a weighted average of the contribution of each adduct.

In Table 1 are listed the unwinding angles for a number of damaging agents. Those studies which have determined the unwinding angles by measuring the shift of the center of the topoisomers Gaussian distribution, and not the shift of the topoisomer bands, are marked with an asterisk (*). As it can be seen, the unwinding angles obtained in conditions of little or no torsional tension range from 6-30° [9, 10, 13-17]. These values are all below the 34.4° unwinding expected for hydrogen bond disruption of one base pair. Therefore, it seems unlikely that, although when formed with groups involved in base pairing, DNA damage does not disrupt cooperative interaction between the flanking regions of base pairs. It suggests instead that it has to accommodate as a stacked intrahelical form. This interpretation is confirmed by the observation that physical binding of a number of planar aromatic carcinogens [15, 18] causes DNA unwind in a fashion similar to known intercalators [19].

Table 1. - *Unwinding angle for modified sites induced by DNA damage*

Damaging agent	Degree/ adduct	Ref.
pyrimidine dimer	8.5	11-13
benzo(a)pyrene-diol-epoxide	30	9
	330 *	9
4,5',8-trimethylpsoralen	28	15
4-nitroquinoline-N-oxide	8.5	*(a)*
N^6-methyladenine	0.5	14
N-acetoxy-2-acetylaminofluorene	20 *	16
	22 *	17
N-acetoxy-2-acetylamino-7-iodofluorene	18	16
cis-$(NH_3)_2PtCl_2$	11	11
	60 *	11
trans-$(NH_3)_2PtCl_2$	6	11

(a) unpublished results

Agarose gel electrophoresis is not only very sensitive to changes in supercoiling but it also responds to changes in length for linear or singly nicked DNA forms. Therefore by looking at the electrophoretic mobility of the nicked RFII form, present as a contaminant in any preparation of circular closed DNA molecules, it is possible to verify whether a DNA lesion modifies helical parameters others than the twist angle between bases. We have observed that high level of UV photoproducts causes DNA to be accelerated [11, 13]. This result suggests that this kind of DNA lesions change the flexibility of the molecule, thus its intrinsic viscosity. An increased mobility of the modified nicked DNA has also been noted in studies with both the *cis*- and *trans*-diamminedichloroplatinum(II) isomers and benzo(a)pyrene diol epoxide [9, 10]. In this case this effect has been explained as due to a decrease in DNA length attributable to local denaturation or microloop formation. However, this interpretation may be controversial in consideration of the small unwinding caused by all these adducts. We think that most likely it results from a decrease in persistence length attributable to reorientation of the helix axis at the site where the lesion is formed.

In vivo analysis of damage induced helical perturbations and effects on DNA topoisomerases

Recognition of DNA damage by repair enzymes is understood in terms of effects of damage on DNA protein interaction. If the perturbation of the DNA structure previously described might be considered as an adequate basis for discrimination between damaged and undamaged sequences by repair proteins, the distortion detected on *in vitro* damaged DNA has to be present also in DNA modified *in vivo*. To answer this question we have carried on band shift experiments on plasmid DNA irradiated within the cell. In Fig. 4 it is shown the effect of UV light on topoisomers originated by relaxation of plasmid pAT153 DNA extracted from UV irradiated cells. It can be seen that UV photodamage changes the electrophoretic mobility of each band in a dose dependent manner analogous to that observed for *in vitro* irradiated topoisomers. These results are suggestive of the fact that DNA organization into chromatin-like structure does not appreciably change the overall perturbation of the DNA structure produced by damaging agents. Thus, the possibility exists that such structural modifications are biologically significant and act as recognition signals for repair enzymes, as well as they interfere with the activity of the enzymes involved in DNA metabolism.

It is well known that DNA polymerases [20], mammalian DNA methylases [21], restriction enzymes [22] are inhibited by UV damage, depurination and alkylation. We have shown that also bacterial type I DNA topoisomerases are inhibited by UV photodamage.

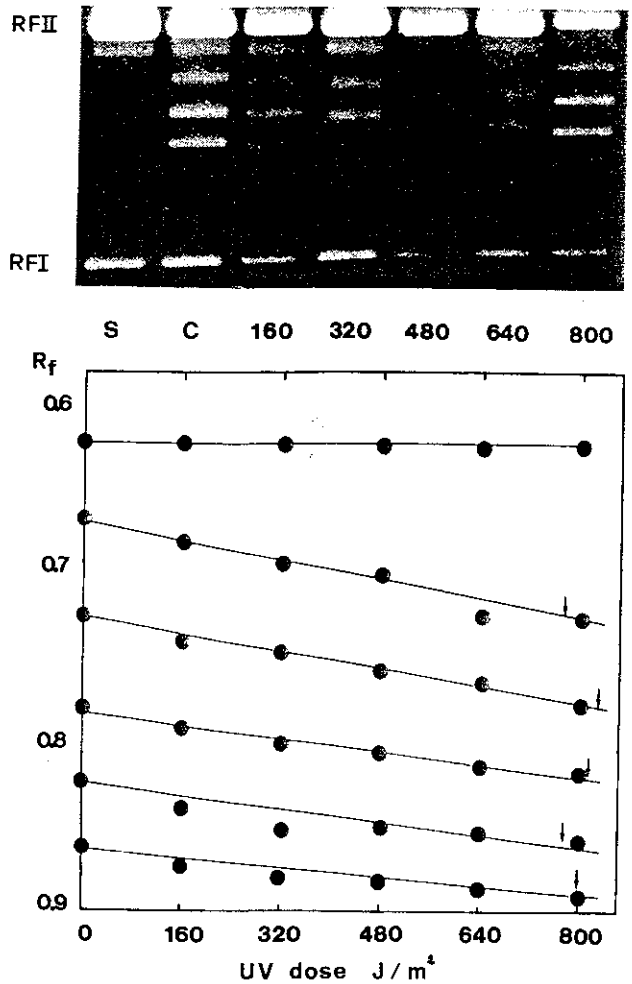

Fig. 4. - Electrophoretic mobility of positively supercoiled topoisomers obtained by relaxation with eukaryotic DNA topoisomerase I of plasmid pAT153 DNA extracted from UV irradiated *E. coli* cells; effect of increasing UV doses on their relative mobility (R_f).

The inhibition is detected as a progressive reduction in the amount of relaxed products, and at UV doses generating less than one pyrimidine dimer per genome. UV damage not only inhibits the activity but also alter the mode of action of these enzymes. In fact, one sees that DNA topoisomerase I shifts from a distributive to a processive mode of action when DNA substrate is UV irradiated [23].

In vitro inhibition of DNA topoisomerase I might be indicative of the significance of this enzyme in phenomena affecting cell recovery from UV damage. In fact, DNA topoisomerase I inhibition could favour DNA gyrase activity over DNA topoisomerase I, thus modulating the expression of genes involved in repair processes or modifying DNA that acts as substrate for repair enzymes. Alternatively *in vivo* inhibition of this enzyme might slow down the advancement of the replication fork providing repair processes with enough time to remove DNA damage.

A relevant role of DNA topoisomerase I in cell recovery from UV damage, is indicated by the UV sensitivity of the *E. coli* strain DM800. This strain is a DNA topoisomerase I *(top A)* deletion mutant, with lower than normal level of supercoiling due to an additional compensatory mutation in the DNA gyrase *(gyr B)* gene. We have shown that the UV sensitivity of this strain probably does not originate from an altered expression of repair enzymes due to the reduction in chromosome supercoiling caused by the *gyr B* mutation, since transfer of the *gyr B* mutation in a wild type background restores the UV resistance. Most likely the UV sensitivity of DM800 is a post-replication repair dependent phenomenon. In fact, a *rec A* mutation cancels the UV sensitivity of the DM800 strain. This suggestion is strengthened by the observation that UV irradiation affects net DNA synthesis and chromosome stability in a way analogous to that reported for mutants altered in functions involved in post-replication repair. This result indicates that DNA topoisomerase I is directly involved in those cellular responses elicited by the proximity of DNA damage to the replication fork [24].

Although we are beginning to have a glimpse of the possible role in DNA repair of the chromosome structure and of the enzymes responsible for its maintenance, a number of questions remains unanswered. For example, we still don't know the nature of the DNA deformation recognized by the repair enzymes, and the role of the superhelical tension in this process. Nor do we know whether DNA supercoiling changes during excision repair, whether the "repairosome" holds during the excision process the incised DNA in a supercoiled state with a mechanism similar to that described for DNA topoisomerases. Finally, we would like to know the role of superhelical tension in the induction of repair enzymes as suggested by the known effects of DNA gyrase inhibitors on the expression of the *rec A* protein.

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