MAMMALIAN DNA-REPAIR ENDONUCLEASE ACTS ONLY ON SUPERCOILED DNA

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1. Introduction

A number of DNA repair enzymes have now been described in mammalian cells [1]. We have isolated and characterized a DNA-repair endonuclease from mouse plasmacytoma cells [2,3] which recognizes minor UV-damage, possibly thymine glycols, OsO_4 and γ-irradiation-induced lesions in DNA. A weak AP-endonucleolytic activity is also associated with this DNA repair enzyme. Recently, we have studied the substrate specificity of the DNA-repair endonuclease in more detail. We report here on the action of the enzyme on UV-irradiated DNAs of different conformations such as regular supercoiled DNA, relaxed covalently-closed circular DNA (rccDNA), linear DNA and denatured DNA. The enzyme was found to be active only on UV-irradiated supercoiled DNA.

2. Materials and methods

2.1. *Preparations of DNAs*

[*methyl*-^{3}H]Thymidine-labelled ϕ X 174 RFI DNA was prepared as in [2]. The DNA was purified by ethidium–CsCl isopycnic centrifugation which was repeated 2 or 3 times. UV-irradiation of the DNA was carried out by use of a Sylvania G8T5 germicidal lamp with an incident dose of 1.4 J . m^{-2} . s^{-1}. The UV-doses employed are indicated below each figure. Relaxed covalently-closed circular DNA was prepared from ϕ X 174 RFI DNA by the use of the nicking–closing enzyme. The reaction mixture contained the following: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol, 100 mM NaCl, 0.04 μg UV-irradiated ϕ X 174 RFI [^{3}H]DNA, 5 μl nicking–closing extract in a total volume of 80 μl. The reaction mixture was kept at 37°C for 15 min, then heated to 70°C for 2 min to inactivate the enzyme.

Linear ϕ X 174 double-stranded DNA was prepared from ϕ X 174 RFI DNA by treatment with the *AvaI* restriction endonuclease. The reaction mixture contained: 6 mM Tris–HCl (pH 7.6), 60 mM NaCl, 6 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 1.5 μg ϕ X 174 [^{3}H]RFI and 3 units *AvaI* in a total volume of 20 μl. The incubation period was 20 min at 37°C. At the end of this period the reaction mixture was heated to 70°C for 2 min to inactivate the enzyme.

2.2. *Enzymes*

DNA-repair endonuclease from mouse plasmacytoma cells was purified as in [2]. The nicking–closing enzyme was prepared from L-cells. Nuclear extracts from L-cells were prepared as previously described and kept at –20°C in 50% glycerol. The extract was found to contain a high nicking–closing activity and low DNA-repair endonuclease activity. *AvaI* restriction enzyme was purchased from BioLab.

3. Results

The action of DNA-repair endonuclease on the UV-irradiated supercoiled form of ϕ X 174 RFI, and rccDNA of this DNA is shown in fig.1. The reaction mixtures were analyzed by the use of the alkaline sucrose gradient technique. In the UV-irradiated control sample, ~30% of the counts were found at the position of the nicked RFII form (fig.1A). Larger

Abbreviations: rccDNA, relaxed covalently closed circular DNA; RFI, replicative form I of ϕ X 174 which is a supercoiled double-stranded molecule; RFII, the nicked form of RFI; AP-sites, apurinic/apyrimidnic sites in DNA

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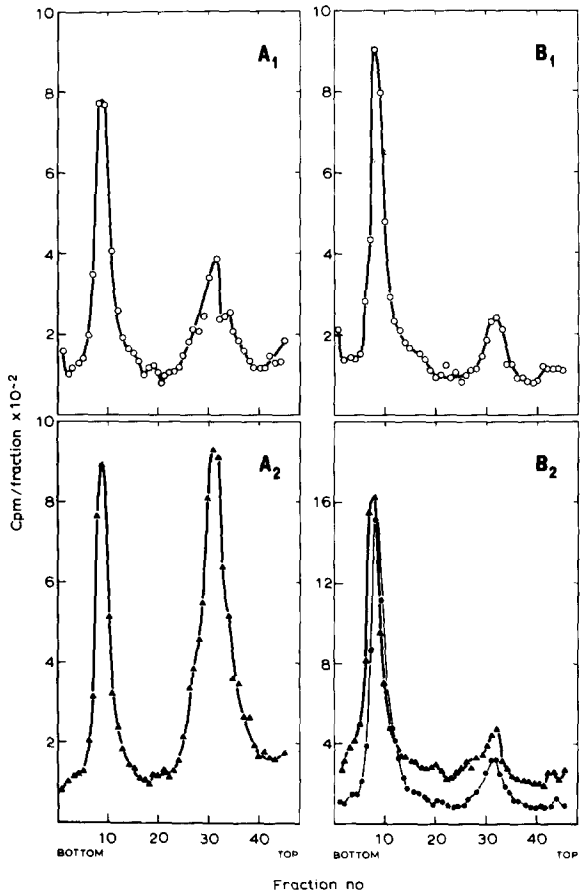

Fig.1. The effect of DNA-repair endonuclease on super-coiled (A) and rccDNA (B) of ϕ X 174 DNA.

(A) DNA-repair endonuclease from mouse plasmacytoma cells and ϕ X 174 RFI [^{3}H]DNA were prepared as in [2,3]. The reaction mixtures contained: 0.04 μg ϕ X 174 RFI [^{3}H]DNA which had been irradiated with a UV-dose (254 nm) of 1.2 kJ/m^2, 10 mM EDTA, 10 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM β-mercaptoethanol and 0.1 units of DNA-repair endonuclease in a total volume of 100 μl. The incubation period was 20 min at 37°C. The reaction was terminated by addition of 40 μl 1 M NaOH, then applied to an alkaline sucrose gradient, 5–20% sucrose, 0.3 M NaOH, 0.7 M NaCl, and 5 mM EDTA. Each tube had a shelf of 0.15 ml 50% sucrose saturated with CsCl. The centrifugations were carried out using an International centrifuge with swingout rotor 498 for 60 min at 60 000 rev./min and at 20°C. The fractions were harvested from the bottom and counted in a dioxane-based scintillation liquid in a Packard 460 CD scintillation counter: A_1 (o——o) control minus DNA-repair endonuclease; A_2 (▲——▲) control plus 0.1 units DNA-repair endonuclease.

(B) The reaction mixtures, gradients and centrifugation times were the same as in (A), except that ϕ X 174 rcc[^{3}H]DNA was used as substrate: B_1 (o——o) control minus DNA-repair endonuclease; B_2 (▲——▲) control plus 0.1 units DNA-repair endonuclease; (●——●) control, unirradiated rccDNA + 0.1 units DNA-repair endonuclease.

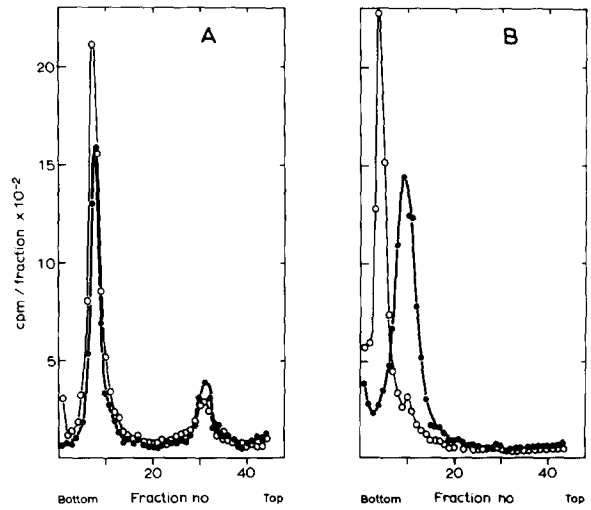

Fig.2. Centrifugation of unirradiated rccDNA in alkaline (A) and neutral (B) sucrose gradient. The rccDNA was prepared as described below fig.1. The alkaline sucrose gradient centrifugations were carried out as given below fig.1. The neutral gradient contained: 5–20% sucrose, 1 M NaCl, 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA; total volume 3.2 ml. Each tube contained 0.15 ml shelf consisting of 50% sucrose saturated with CsCl. The centrifugations were carried out with the IEC rotor 498 for 3 h at 60 000 rev./min and at 20°C: (o——o) ϕ X 174 [^{3}H]RFI control; (●——●) ϕ X 174 [^{3}H]rccDNA.

UV-doses lead to progressively more of this form. Of the amount of RFII in the gradient, ~1/3 was due to the RFII present in the unirradiated control, (fig.2A). The remaining 2/3 of the RFII peak was formed by UV-irradiation and subsequent alkaline sucrose gradient centrifugation. Close to 1/3 of the counts in the RFII peak is due to alkaline labile AP-sites and another 1/3 to alkaline sensitive minor UV products of unknown chemical composition. The number of AP-sites were estimated by the use of a separate AP-endonuclease [4] and also by a newly developed polyamine method (R. Male and K. Kleppe, unpublished). The UV-irradiated ϕ X 174 RFI DNA was readily converted to the RFII DNA by the DNA-repair endonuclease (fig.1 A_2). Taking into account the alkaline hydrolysis of the control sample described above, it can be estimated that an enzymatic conversion of ~50% from RFI to form RFII has taken place with the present UV-dose and amount of enzyme used. No hydrolysis was detected with the unirradiated DNA (results not shown). When the same amount of enzyme and the same UV-dose were employed with rccDNA of ϕ X 174, virtually no conversion to the RFII form

was detected (fig.1B). The rccDNA was prepared from ϕ X 174 RFI form, using the nicking–closing enzyme from L-cells [5]. UV-irradiation of the DNA prior to or after the nicking–closing enzyme treatment had no influence on the results. The fact that rccDNA was obtained was proven by agarose gel electrophoresis and centrifugation in alkaline and neutral sucrose gradients (fig.2). As expected there was no difference in the sedimentation rate between the two DNA forms in the alkaline sucrose gradient (fig.2A). The results also indicate that essentially no nicks have been introduced during the nicking–closing reaction. In the neutral gradient, on the other hand, the rccDNA sedimented with an S-value of 17 S *vs* 21 S for the supercoiled RFI form (fig.2B).

The action of DNA-repair endonuclease on linear DNA was also investigated. ϕ X 174 RFI DNA was linearized by the action of the *Ava*I restriction endonuclease which makes one cut in ϕ X 174 RFI [6]. When this DNA was irradiated with UV-light and incubated with DNA-repair endonuclease, no breaks in the DNA were detected (fig.3). The results were the same whether the DNA was irradiated prior to or after the *Ava*I treatment. Similar results were also obtained when linear UV-irradiated λ-DNA was employed as a substrate (not shown).

Experiments were also carried out with UV-irradiated single-stranded DNAs. The DNAs employed were alkali denatured ϕ X 174 RFII and *Ava*I-treated ϕ X 174 RFI. No hydrolysis was detected when these UV-irradiated DNAs were incubated with DNA-repair endonuclease and the reaction mixtures subsequently analyzed by the alkaline sucrose gradient technique.

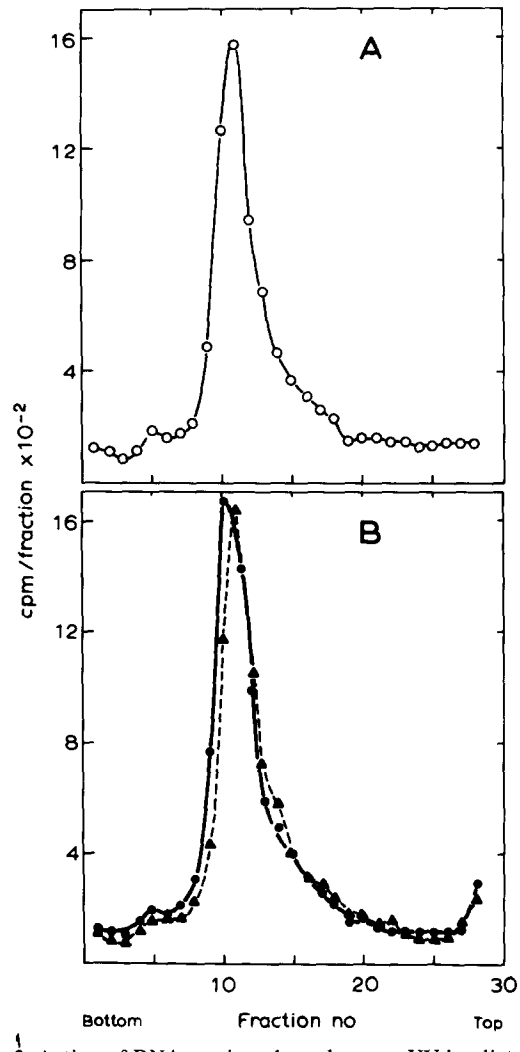

Fig.3. Action of DNA-repair endonuclease on UV-irradiated linear double-stranded ϕ X 174 DNA. ϕ X 174 [^{3}H]RFI was treated with *Ava*I restriction endonuclease as described in section (2). UV-irradiation was carried out prior to the enzyme treatment using a dose of 1.2 kJ/m^2 (254 nm). The reaction mixtures for the DNA-repair endonuclease treatment were the same as described below fig.1 except that *Ava*I-treated DNA was used, and the alkaline sucrose gradient centrifugations were carried out for 165 min: (A) (o——o) control minus DNA-repair endonuclease; (B) (▲——▲) control plus 0.02 units DNA-repair endonuclease; (●——●) control plus 0.1 units DNA-repair endonuclease.

4. Discussion

These results strongly suggest that under the present experimental conditions the DNA-repair endonuclease incised only damaged double-stranded DNA in its superhelical form and not when the damaged DNA was relaxed, linear or single-stranded. The data cannot be explained on the basis that UV-light causes different bases to be modified in the various DNAs since UV-irradiation prior to or after preparation of the DNAs from the RFI did not affect the results. The enzyme must therefore recognize a particular structural feature of the superhelicity in addition to the lesion in DNA. It has been known for some time that supercoiled plasmid DNA contain regions which are susceptible to single-stranded specific nucleases. In the case of the two supercoiled DNAs, pVH51 and pBR322, site-specific cleavage by S_1 single-stranded and T_7 nuclease takes place in potential cruciform structures [7]. With other DNAs, including ϕ X 174 RFI, the cleavage sites of these enzymes are located more randomly, despite the fact that specific cruci-

form structures also could form in these DNAs from inverted repeat sequences. Possible semi-stable cruciform structures could also be present in A + T rich regions in these DNAs [7]. UV-damaged thymine residue, whether it be a thymine dimer or thymine glycol, would be expected to have a greatly reduced capacity for hydrogen binding to adenine residues. Thus Denhardt and Cato [8] have shown that when ϕ X 174 RFI is UV-irradiated with doses ~10-times larger than those described here the DNA molecule becomes partially untwisted. Damaged base residues could also constitute possible nuclei for the formation of cruciform structures in supercoiled DNAs. One possible explanation for the observations here is that the DNA-repair endonuclease binds to unique structures in the supercoiled DNA which contain lesions and then makes a nick at or in such a damaged structure. Once a nick has been introduced in the supercoiled DNA, the DNA immediately reverts to the relaxed form, RFII, and since this is not a substrate for the enzyme, no further nicking will take place.

The question can then be raised whether there is a separate repair endonuclease for damaged relaxed DNAs. Such an enzyme could exist since only supercoiled DNA has been used in the assay for the enzymatic activity. Another possibility is that a certain protein factor needed for recognition of relaxed DNAs has been lost during the purification. We are currently investigating these aspects using rccDNA as a substrate.

In most experiments with the DNA-repair endonuclease where supercoiled DNA has been employed as a substrate, a Poisson distribution has been employed in the estimation of the number of nicks per DNA molecule. The results obtained in this work suggest that this can no longer be applied to this enzyme since it will make only one nick per DNA molecule. The number of nicks per DNA molecule given in earlier reports have been therefore somewhat overestimated.

These data suggest that superhelicity in DNA may be an important aspect in many DNA repair systems. The different allomorphic forms of the supercoiled DNA should be employed as substrate in assays for repair enzymes. More information about specific changes in the helical structure of supercoiled DNAs induced by irradiation and by specific chemicals are also required before a full understanding of the mechanism of action of many repair enzymes can be given.

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References

[1] Hanawalt, P. C., Cooper, P. K., Ganesan, A. K. and Smith, A. (1979) Annu. Rev. Biochem. 48, 783–836.
[2] Nes, I. F. and Nissen-Meyer, J. (1978) Biochim. Biophys. Acta 520, 111–121.
[3] Nes, I. F. (1980) Eur. J. Biochem. 112, 161–168.
[4] Nes, I. F. (1980) Nucleic Acids Res. 8, 1575–1590.
[5] Male, R., Nes, I. F. and Kleppe, K. (1981) Eur. J. Biochem. 121, 243–248.
[6] Murray, K., Hughes, S. G., Brown, J. S. and Bruce, S. A. (1976) Biochem. J. 159, 317–322.
[7] Panayotos, N. and Wells, R. D. (1981) Nature 289, 466–470.
[8] Denhardt, D. T. and Kato, A. C. (1973) J. Mol. Biol. 77, 479–494.