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Repair of depurinated DNA with enzymes from rat liver chromatin

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DNA from T7 phage containing AP (apurinic/apyrimidinic) sites was repaired by the successive actions of three chromatin enzymes [AP endodeoxyribonuclease, DNAase IV (5′→3′-exodeoxyribonuclease) and DNA polymerase-β] prepared from rat liver and T4-phage DNA ligase. Since DNA ligase is also found in rat liver chromatin, all the activities used for the successful repair *in vitro* are thus present in the chromatin of a eukaryotic cell. Our results show, in particular, that the chromatin DNAase IV is capable of excising the AP site from the DNA strand nicked by the chromatin AP endodeoxyribonuclease. We did not try to combine all the enzymes, since competition between some of them might have prevented the repair; we have, for instance, shown that DNA ligase can seal the incision 5′ to the AP site made by the AP endodeoxyribonuclease. Changes in chromatin structure during repair might perhaps prevent this competition when nuclear DNA is repaired in the living cell.

The mechanism used by *Escherichia coli* to repair AP (apurinic or apyrimidinic) sites in DNA has been known for several years (Gossard & Verly, 1978). How the same DNA lesions are repaired in eukaryotic cells is still uncertain; one aspect of this question is whether or not all the necessary factors are in chromatin. It is usually believed that the repair of AP sites begins with the incision of the damaged DNA strand by an AP endodeoxyribonuclease. César & Verly (1983) have purified the AP endodeoxyribonuclease of rat liver chromatin and shown that it hydrolyses the phosphodiester bond 5′ to the AP site, leaving 3′-hydroxy and 5′-phosphate ends. The next question was whether rat liver chromatin also contained a 5′→3′-exonuclease capable of excising the AP site from the nicked DNA strand. Germaine Zocchi (Zocchi, 1984) in our laboratory had shown that DNAase IV, which is an exonuclease degrading double-stranded DNA from nicks in the 5′→3′ direction (Lindahl *et al.*, 1969), is present in rat liver chromatin; nobody knew, however, if an AP site would not be an insuperable obstacle for this exonuclease.

In preliminary work (Goffin & Verly, 1982), we made the qualitative demonstration of the excision of AP sites from super-twisted RF-I DNA of phage ϕX174 by the conjugated actions of the chromatin AP endodeoxyribonuclease and the chromatin DNAase IV. To quantify better the repair of AP sites, we decided to perform a similar experiment with T7-phage DNA. With this linear double-stranded DNA, it is possible to count, with a good accuracy, an average of 0.1 to 13 breaks per strand of 39936 nucleotides by using centrifugation in neutral-sucrose gradients and computer analysis of the sedimentation profiles. Moreover, pre-existing strand breaks and intact AP sites (i.e. AP sites not associated with strand breaks) can easily be distinguished and counted separately (Crine & Verly, 1976). Our results show that linear double-stranded T7-phage DNA containing AP sites is completely repaired by the successive actions of three enzymes extracted from rat liver chromatin [AP endodeoxyribonuclease (EC 3.1.25.2), DNAase IV, DNA polymerase-β (EC 2.7.7.7)] and T4-phage DNA ligase (EC 6.5.1.1). Thus DNAase IV can excise the AP site from the DNA strand nicked by the AP endodeoxyribonuclease, and the resulting gap can be filled by DNA polymerase-β; the final ligation restores the intact DNA molecule. Since rat liver chromatin also contains a DNA ligase, the conclusion is that all the factors necessary to repair DNA containing AP sites along the suggested pathway are present in the chromatin of a eukaryotic cell.

Abbreviations used: AP, apurinic or apyrimidinic; DNAase, deoxyribonuclease; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid.

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Materials and methods

Enzymes

The AP endodeoxyribonuclease, the 5′→3′-exodeoxyribonuclease (DNAase IV) and the DNA polymerase-β were prepared from rat liver chromatin as described by Goffin & Verly (1982). The three enzymes were kept in buffer A (20mM-Tris/HCl/1mM-EDTA/0.2mM-dithiothreitol/0.04% bovine serum albumin, pH8.0) containing glycerol. T4-phage DNA ligase was purchased from Bethesda Research Laboratories, Gaithersburg, MD 20877, U.S.A. (B.R.L.); it was diluted in buffer B (40mM-Hepps/12mM-$MgCl_2$/1mM-dithiothreitol/0.04% bovine serum albumin, pH8.0). The enzymes units are as defined previously (Goffin & Verly, 1982).

Substrates

'Normal' [^{3}H]DNA. ^{3}H-labelled phage-T7 DNA, with a specific radioactivity of 329000 d.p.m./μg, was prepared as described by Verly *et al.* (1974). It was kept at 4°C in 0.15M-NaCl/0.015M-EDTA (pH7.0)/0.1% benzyl alcohol. It contained an average of 0.24 break per strand of 39936 nucleotides and no intact apurinic sites.

Depurinated [^{3}H]DNA. To 1vol. of [^{3}H]DNA solution (340μg/ml) was added 1vol. of 0.5M-acetate buffer so that the final pH was 4.0, and the mixture was incubated for 50 or 90min at 37°C depending on the experiment. The depurinated [^{3}H]DNA species were used on the same day; their contents of breaks and intact AP sites are indicated in Table 1.

Incubation of the depurinated [^{3}H]DNA with the repair enzymes

The depurinated [^{3}H]DNA was dialysed four times for 1h against 5mM-NaCl/1mM-sodium citrate/0.1mM-EDTA, pH8.0. To 360μl of the dialysed solution containing 51μg of depurinated [^{3}H]DNA were added 140μl of 200mM-Hepps/60mM-$MgCl_2$/8mM-dithiothreitol/0.4mM-EDTA, pH8.0, and 60μl of AP endodeoxyribonuclease (1380 units) solution. A control without AP endodeoxyribonuclease was also prepared. Both were incubated for 15min at 37°C.

Tubes were prepared that received 60μl of depurinated [^{3}H]DNA, treated or not with the AP endodeoxyribonuclease, and 10μl of buffer A with or without 0.32 unit of 5′→3′-exodeoxyribonuclease. After a 30min incubation at 37°C, the two enzymes (AP endodeoxyribonuclease and 5′→3′-exodeoxyribonuclease) were inactivated by heating for 5min at 65°C.

The tubes then received 10μl of 4.8mM-ATP, 200mM-Hepps, 60mM-$MgCl_2$, 8mM-dithiothreitol, 0.4mM-EDTA, pH8.0, 10μl of water containing the four deoxynucleoside triphosphates (0.48mM each), 20μl of buffer A with or without 0.76 unit of

Table 1. *Repair of depurinated T7-phage DNA*

The depurinated [^{3}H]DNA was incubated with the AP endodeoxyribonuclease, then with the 5′→3′-exodeoxyribonuclease, before heat-inactivation of both enzymes; DNA polymerase-β with the four deoxyribonucleosides triphosphates and T4-phage DNA ligase with ATP were added and the mixture further incubated at 15°C for 2h, except for Expt. I, no. 8 (3h). The DNA samples were denatured with formamide, pH7.0, or with NaOH followed by neutralization, before determination of the number of breaks by neutral-sucrose-gradient analysis. Abbreviations used: n = average number per T7-phage DNA strand, of breaks and/or intact AP sites; Endo, chromatin AP endodeoxyribonuclease; Exo, chromatin 5′→3′-exodeoxyribonuclease; Pol, chromatin DNA polymerase-β; Lig, T4-phage DNA ligase.

Expt. no.	Treatment no.	Endo	Exo	Pol	Lig	n: Breaks NaOH	n: Breaks Formamide	n: Intact AP sites
I	1	−	−	−	−	6.46	1.59	4.87
	2	+	−	−	−	6.68	6.06	0.62
	3	+	−	−	+	5.48	2.44	3.04
	4	+	+	−	−	6.78	7.28	0.00
	5	+	+	−	+	6.32	6.02	0.30
	6	+	+	+	−	7.80	lost	
	7	+	+	+	+	2.18 2.20 2.26 2.21	2.34	0
	8	+	+	+	+	2.14	2.22	0
II	1	−	−	−	−	4.30	1.30	3.00
	2	+	+	+	+	1.72	1.56	0.16

DNA polymerase-β, and 20μl of buffer B with or without 7.2 units of T4-phage DNA ligase. After a 2 or 3h incubation at 15°C, the reactions were stopped by addition of 20μl of 0.2M-EDTA, pH8.0, and 20μl of 1% (w/v) sodium dodecyl sulphate, followed by 2min heating at 65°C.

The volume in each tube was adjusted to 400μl and the KCl and EDTA concentrations to 0.5M and 15mM respectively; the proteins were extracted with phenol and the aqueous phase dialysed for 2h against 0.5M-NaCl/15mM-EDTA, pH8.0, then overnight against 15mM-NaCl/15mM-EDTA, pH7.0. Each sample was subsequently divided in two for denaturation either with formamide at pH7.0, or with NaOH followed by neutralization. The denatured DNA species were then analysed by sedimentation on neutral-sucrose gradients.

Denaturation of DNA by NaOH or formamide and estimation of the number of breaks and intact AP sites per strand

The method was described by Verly *et al.* (1974). The depurinated [^{3}H]DNA was denatured in two ways: either with formamide at neutral pH, which left the AP sites intact, or with NaOH followed by neutralization, which placed a break near each AP site. The separated strands were then centrifuged on neutral-sucrose gradients, which were fractionated for radioactivity measurements; the number of breaks was calculated from the sedimentation profile by using a computer, with the profile of intact DNA strands as a reference. When the formamide-denatured DNA is analysed, one obtains the average number of breaks per strand that were present in the double-stranded DNA. When the alkali-denatured DNA is analysed, one obtains the sum of pre-existing breaks and those resulting from the NaOH treatment. The difference between the two results is the average number of intact AP sites per strand that were present in the double-stranded DNA (Crine & Verly, 1976).

It is important to note that, with time, AP sites lead to interstrand cross-links resistant to formamide (Goffin & Verly, 1983); partial renaturation when the formamide is removed disturbs the sedimentation profiles. To limit this difficulty to a minimum, the depurinated DNA was used immediately.

Results

Two independent experiments (I and II) were carried out. The results are shown in Table 1.

In Expt. I the depurinated T7-phage [^{3}H]DNA incubated without any enzyme (treatment no. 1, Table 1) contained an average of 1.59 breaks and 4.87 intact AP sites per strand (39936 nucleotides). An incubation with the AP endodeoxyribonuclease (no. 2) hydrolysed a phosphoester bond near most of the intact AP sites (their number was decreased from 4.87 to 0.62); the enzyme was specific for AP sites, since the number of lesions (breaks + intact AP sites) counted after NaOH denaturation did not change significantly (6.46 and 6.68).

A treatment with T4-phage DNA ligase sealed the incisions made by the AP endodeoxyribonuclease; the number of intact AP sites, which was 0.62 per strand after the AP endodeoxyribonuclease treatment and inactivation of this enzyme (no. 2), was raised to 3.04 by T4-phage DNA ligase (no. 3).

An incubation with the 5′→3′-exodeoxyribonuclease, which widened into gaps most of the incisions made by the AP endodeoxyribonuclease, prevented the ligase action; the decrease in total lesions measured after NaOH denaturation and the reappearance of intact AP sites were negligible (compare treatment no. 4 with no. 5).

A treatment with DNA polymerase-β alone after the exonuclease (no. 6) left a nick that could be sealed by ligase (no. 7). With no. 7, four different analyses were performed after denaturation with NaOH; the results show the good reproducibility of the method for counting breaks in the DNA strands. After the incubation with the four enzymes, no intact AP sites were left and the number of total lesions, which were thus only breaks, was decreased from 6.46 to about 2.20 per strand. Increasing the period of incubation with DNA polymerase-β and ligase from 2 to 3h at 15°C did not increase the repair significantly (no. 8).

Comparing treatments no. 8 and no. 1 enables us to calculate the number of AP sites, intact in the substrate DNA, that were repaired. The substrate contained an average of 6.46 lesions per strand, of which 4.87 were intact AP sites; after incubation with the four enzymes, there remained 2.14 lesions, none of which was an intact AP site. Two different hypotheses will set the limits of the repair. If the repair of breaks had an absolute priority, the remaining lesions should derive from not-completely-repaired AP sites; following this hypothesis, the average number of intact AP sites that would have been repaired is: $4.87-2.14=2.73$ per strand. If it was the repair of intact AP sites that had an absolute priority, the average number of intact AP sites which would have been repaired is: $6.46-2.14=4.32$ per strand. The conclusion is thus that, of the 4.87 intact AP sites per strand in the depurinated DNA substrate, from 2.73 to 4.32 were repaired in our system *in vitro*.

Expt. II leads to a similar conclusion: of the 3.00 intact AP sites per strand that were in the substrate, from 1.28 to 2.58 were repaired. Fig. 1 presents the sedimentation profiles, after NaOH

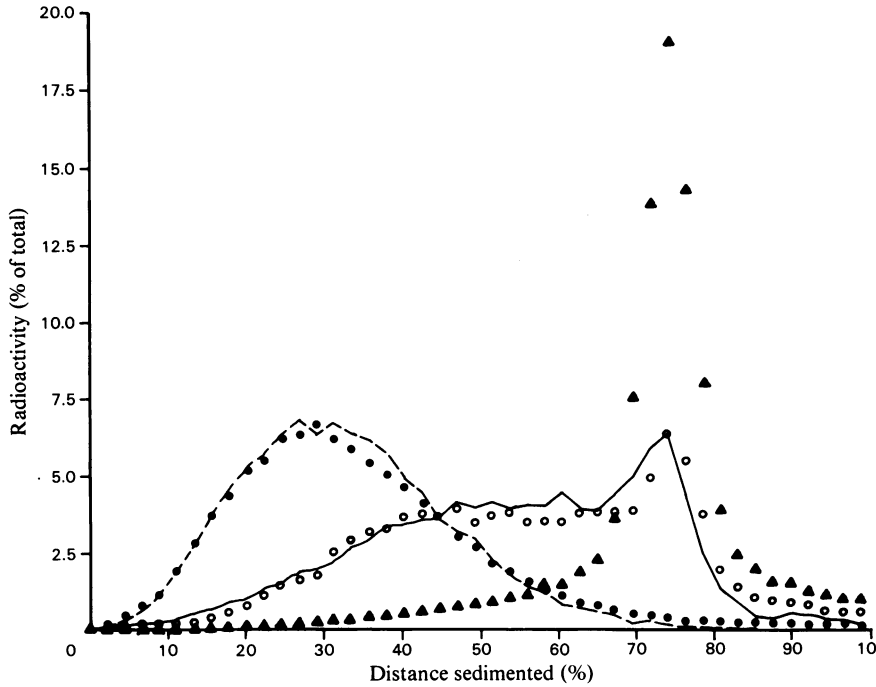

Fig. 1. *Sedimentation profiles of depurinated T7-phage DNA, repaired and unrepaired*
Depurinated T7-phage [^{3}H]DNA was incubated successively with the AP endodeoxyribonuclease, the 5′→3′-exodeoxyribonuclease and DNA polymerase-β of rat liver chromatin, and T4-phage DNA ligase (○); a control was incubated without enzyme (●) (Expt. II of Table 1). The DNA samples were then denatured with NaOH and the solutions neutralized before centrifugation on neutral-sucrose gradients. The 5 ml gradients were emptied from the top and the radioactivities of the 45 collected fractions were determined (% of total radioactivity); the abscissa gives the distance sedimented as a percentage of the height of the gradient. The profile used as a reference (▲) corresponds to the sedimentation of intact T7-phage DNA strands. The continuous and discontinuous lines are theoretical curves, drawn by the computer, that best fitted the experimental data; for the DNA incubated without enzymes (discontinuous line), it is representative of an average of 4.30 lesions per strand (1.30 breaks + 3.00 intact AP sites; see Table 1); for the DNA incubated with the enzymes (continuous line), it is representative of an average of 1.72 lesions per strand (1.56 breaks + 0.16 intact AP sites).

denaturation, of the depurinated [^{3}H]DNA incubated with or without the four enzymes; the reappearance of intact DNA strands when the enzymes were present during the incubations illustrates the repair.

Discussion

The chromatin AP endodeoxyribonuclease from rat liver is a 5′-AP endodeoxyribonuclease; it hydrolyses the phosphodiester bond that is the immediate neighbour of the AP site on its 5′-side, leaving 3′-OH and 5′-phosphate ends (César & Verly, 1983). DNAase IV is an exonuclease working on double-stranded DNA from nicks in the 5′→3′ direction (Lindahl *et al.*, 1969); this enzyme has been found in rat liver chromatin, and the question was whether, after incision on the 5′-side of the AP site by the AP endodeoxyribonuclease, this 5′→3′-exodeoxyribonuclease was able to excise the AP site. The results presented here, as well as those previously obtained with phage-ϕX174 RF (replicate form) DNA (Goffin & Verly, 1982), indicate that the AP site is indeed excised.

A DNA polymerase is needed to fill the gap resulting from the exonucleolytic degradation of the DNA strand that carried the AP site. DNA polymerase-α and DNA polymerase-β are involved in DNA repair (Miller & Chinault, 1982); it seems, however, that DNA polymerase-α needs a large gap to place itself and that DNA polymerase-β is always necessary to complete the filling (Wang & Korn, 1980). Since it is not necessary to remove many nucleotides to get rid of the AP site in our system *in vitro*, the use of DNA polymerase-β seemed mandatory.

In our experiments, the removal of the AP site

by the 5′→3′-exodeoxyribonuclease preceded the treatment with DNA polymerase-β; the displacement of the strand by the polymerase, which is favoured by the presence of an AP site at its 5′-end, and the resulting abortion of the repair (Mosbaugh & Linn, 1982), were thus unlikely. Nevertheless, to decrease the possibility of such an occurrence as much as possible, the incubation with DNA polymerase-β was carried out at low temperature and in the presence of DNA ligase.

We used T4-phage DNA ligase and, making the hypotheses that breaks were repaired before the intact AP sites or vice versa, we could calculate the minimum and maximum numbers of AP sites, intact in the substrate DNA, that had disappeared after the successive actions of the four enzymes, leaving normal continuous strands. We also performed an experiment with DNA ligase prepared from rat liver chromatin (results not shown), but we used 100 times less enzyme; we observed some repair, but not enough to prove unambiguously that some AP sites were repaired.

This quantitative work on T7-phage DNA thus completely confirms the qualitative one previously published, where we used phage-ϕX174 RF DNA (Goffin & Verly, 1982). Rat liver chromatin possesses all the enzymes necessary to repair DNA containing AP sites according to the following model: the AP endodeoxyribonuclease cuts the strand 5′ to the AP site; the 5′→3′-exodeoxyribonuclease excises the AP site and perhaps a few nucleotides; DNA polymerase-β fills the gap, and ligase terminates the repair. This seems not to be the only possible repair pathway; Mosbaugh & Linn recently (1983) proposed three other models in which the AP site is excised either by the joint action of a 3′ AP endodeoxyribonuclease and a 5′ AP endodeoxyribonuclease, or by a DNA polymerase-β–DNAase V complex after the DNA has been incised either by a 3′ or a 5′ AP endodeoxyribonuclease. Nobody knows if these models can be extrapolated to the repair of nuclear DNA *in vivo*. The search for a 3′ AP endodeoxyribonuclease in the non-histone proteins of rat liver chromatin has so far been unsuccessful.

Comparison of treatments no. 1, 2 and 3 in Expt. I (Table 1) clearly shows that the incision made by the chromatin AP endodeoxyribonuclease can be sealed with a DNA ligase, restoring an intact AP site. Since the chromatin AP endodeoxyribonuclease hydrolyses the phosphodiester bond immediately on the 5′-side of the AP site, this result means that ligation occurs between the 3′-OH end of a normal polynucleotide and the 5′-phosphate belonging to a deoxyribose residue not carrying a base. Such ligation and the resulting abortion of the repair could not occur in our complete system *in vitro*, since the incision made by the AP endodeoxyribonuclease was enlarged by the 5′→3′-exodeoxyribonuclease before the addition of DNA ligase.

One must underline the multiple oppositions between the mammalian enzymes which are likely to play a rôle in the repair of AP sites: we mentioned above the competition between ligase and the 5′→3′-exodeoxyribonuclease, but there is also one between the 5′→3′-exodeoxyribonuclease that degrades DNA and the DNA polymerase-β that resynthesizes it. These interferences do not exist with *E. coli* enzymes: endonuclease VI is, at the same time, a 5′ AP endodeoxyribonuclease and a 3′→5′-exodeoxyribonuclease (exonuclease III) that works as an anti-ligase; the concerted polymerase and 5′→3′-exodeoxyribonuclease activities of DNA polymerase I ensure that the excision of the AP site is followed by the translation of a nick that DNA ligase can seal any time (Gossard & Verly, 1978). These warranties given by the dual activities of the bacterial repair enzymes are strikingly absent from the eukaryotic repair enzymes. It might be that, in the living cell, changes in the chromatin structure program the orderly successive actions of the DNA repair enzymes as we did in our model *in vitro*.

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