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Possible roles of β-elimination and δ-elimination reactions in the repair of DNA containing AP (apurinic/apyrimidinic) sites in mammalian cells

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Histones and polyamines nick the phosphodiester bond 3′ to AP (apurinic/apyrimidinic) sites in DNA by inducing a β-elimination reaction, which can be followed by δ-elimination. These β- and δ-elimination reactions might be important for the repair of AP sites in chromatin DNA in either of two ways. In one pathway, after the phosphodiester bond 5′ to the AP site has been hydrolysed with an AP endonuclease, the 5′-terminal base-free sugar 5′-phosphate is released by β-elimination. The one-nucleotide gap limited by 3′-OH and 5′-phosphate ends is then closed by DNA polymerase-β and DNA ligase. We have shown *in vitro* that such a repair is possible. In the other pathway, the nicking 3′ to the AP site by β-elimination occurs first. We have shown that the 3′-terminal base-free sugar so produced cannot be released by the chromatin AP endonuclease from rat liver. But it can be released by δ-elimination, leaving a gap limited by 3′-phosphate and 5′-phosphate. After conversion of the 3′-phosphate into a 3′-OH group by the chromatin 3′-phosphatase, there will be the same one-nucleotide gap, limited by 3′-OH and 5′-phosphate, as that formed by the successive actions of the AP endonuclease and the β-elimination catalyst in the first pathway.

INTRODUCTION

The mechanism of the repair of DNA containing AP (apurinic/apyrimidinic) sites is usually supposed to follow the general model of excision repair, where the damaged fragment is released by phosphodiesterases, creating a gap that is filled by DNA polymerase, the sister strand acting as a template; the remaining 'nick' is then closed by DNA ligase. The discovery of AP endonucleases in all the living organisms that have so far been studied (Verly *et al.*, 1973) led one to suppose that the repair of AP sites must be initiated by these enzymes. These AP endonucleases cleave the phosphodiester bond on the base-free deoxyribose 5′ side, giving 3′-OH and 5′-phosphate ends (Gossard & Verly, 1976; Clements *et al.*, 1978; César & Verly, 1983). After their action, the release of the AP site needs a second cleavage on the opposite side. How this second cleavage is performed appears to be different in bacteria and mammalian cells.

Gossard & Verly (1976, 1978) have shown that the second cleavage 3′ to the AP site is carried out by the 5′→3′ exonuclease activity of DNA polymerase-I in *Escherichia coli*. This phosphodiesterase activity hydrolyses the second or third phosphodiester bond 3′ to the AP site; after the action of endonuclease VI (the main AP endonuclease of *E. coli*; also termed 'exonuclease III'), DNA polymerase I quickly releases the AP site as deoxyribose 5-phosphate bound to one or two nucleotides.

Rat liver chromatin contains an AP endonuclease (Verly & Paquette, 1973; Thibodeau & Verly, 1980; César & Verly, 1983) and a 5′→3′ exonuclease (DNAase IV) (Lindahl *et al.*, 1969; Grondal-Zocchi & Verly, 1985). After the action of the AP endonuclease on a DNA containing AP sites, these lesions can be excised by DNAase IV (Goffin & Verly, 1982, 1984), but the excision is very slow (Grondal-Zocchi & Verly, 1985). In contrast with the DNA polymerase I 5′→3′ exonuclease mentioned above, the mammalian enzyme cleaves the phosphodiester bond immediately on the 3′ side of the AP site; moreover, the cleavage is not the result of a hydrolysis but of a β-elimination reaction (Grondal-Zocchi & Verly, 1985). DNAase V from Novikoff hepatoma has both 3′→5′ and 5′→3′ exonuclease activities and it forms a complex with DNA polymerase-β (Mosbaugh & Meyer, 1980). Mosbaugh & Linn (1983) have excised AP sites from DNA by the successive actions of an AP endonuclease and DNAase V; there are no data to show whether the AP site excision was the result of a hydrolysis or of a β-elimination. Mosbaugh & Linn (1980) have described, in human fibroblast extracts, a 3′ AP endonuclease (called 'AP endonuclease I') that cleaves the phosphodiester bond 3′ to AP sites in addition to a more classical 5′ AP endonuclease (called 'AP endonuclease II') that hydrolyses the phosphodiester bond 5′ to AP sites. The combined action of these two AP endonucleases releases the AP sites as sugar-phosphate molecules and leaves one-nucleotide gaps. The exact nature of this mammalian 3′ AP endonuclease has not yet been clarified, because it has not been purified. We have reason to believe that the human fibroblast AP endonuclease I is not an endonuclease but some basic protein catalysing a β-elimination, as we showed to be the case for a bacterial 3′ AP endonuclease (Bailly & Verly, 1987); indeed, Mosbaugh & Linn (1980)

Abbreviations used: Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-propane-3-sulphonic acid; RF, replication form; d(-), a base-free deoxyribose in DNA which is the same as an apurinic/apyrimidinic (AP) site.

observed that partially depurinated DNA, treated with human fibroblast AP endonuclease I, does not support DNA synthesis by *E. coli* DNA polymerase-I, and we have shown that a 3′ terminal base-free deoxyribose is easily removed by the 3′→5′ exonuclease activity of this enzyme (Bailly & Verly, 1984). We conclude that, in mammalian cells, there is not a single known enzyme that has been convincingly shown to hydrolyse a phosphodiester bond 3′ to an AP site. It can thus be hypothesized that the 3′ nicking is, rather, the result of a β-elimination.

In chromatin, numerous molecules can nick DNA strands at AP sites by catalysing a β-elimination: basic proteins, especially histones, and polyamines. One can thus postulate that, in mammalian cells, the excision of AP sites results from the combined actions of a 5′ AP endonuclease and a β-elimination catalyst. The subject of the present study was to check this hypothesis *in vitro* and also to determine the order in which the two reactions can operate.

MATERIALS AND METHODS

Enzymes

Chromatin AP endonuclease from rat liver was purified as described by César & Verly (1983); it was stored at 4 °C in 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/0.2 mM-dithiothreitol/10% (v/v) glycerol.

Rat liver DNA polymerase-β was prepared from chromatin non-histone proteins by two successive affinity-chromatographic runs (one on double-stranded-DNA–cellulose and one on single-stranded-DNA–Sepharose); it was kept at −20 °C in 10 mM-Tris/HCl (pH 8.0)/0.5 mM-EDTA/0.1 mM-dithiothreitol/50% (v/v) glycerol. The activity of the solution, measured as described by Richardson *et al.* (1964) was 26 units/ml.

Phage-T_4 DNA ligase was bought from B.R.L. It was kept at −20 °C in 10 mM-Tris/HCl (pH 7.5)/50 mM-KCl/1 mM-dithiothreitol/50% (v/v) glycerol. The activity was 1.4 units/ml.

Other reagents

Spermine (base) and spermidine (trihydrochloride) were bought from Sigma. They were kept at −20 °C in solution adjusted to pH 7.0 by the addition of 1 M-HCl.

Histones were extracted from rat liver chromatin as described by Richards & Ramsay-Shaw (1982) and purified on Sephadex G-50 (Pharmacia) by the method of Van der Westhuysen & Van Holt (1971); histone H1 was separated from the other histones by chromatography on Bio-Gel P60 (Bio-Rad). It was stored at −20 °C at 0.2 mM concentration in a solution adjusted to pH 7.0 by the addition of HCl.

DNA substrates

5′-^{32}P-labelled oligo(dT) with a base-free deoxyribose [d(-)] instead of the ninth nucleotide {[5′-^{32}P]-pdT$_8$d(-)dT$_n$} was prepared as described by Bailly & Verly (1987).

DNA with AP sites labelled with ^{32}P on their 5′ side and with ^{3}H in the 1′ and 2′ positions of the base-free deoxyribose {[d(-)-1′,2′-^{3}H,5′-^{32}P]DNA} was prepared as described by Bailly & Verly (1987). Its specific radioactivities were 4400 d.p.m. of ^{3}H and 5100 d.p.m. of ^{32}P/μg, and it contained 1 AP site/2000 nucleotides.

Phage ϕX174 RF-I DNA labelled with ^{3}H was prepared by replication, in the presence of [^{3}H]thymidine and chloramphenicol, in *thy*$^-$ *E. coli* C HF 4733 (Axelrod, 1976). It was extracted from the crude extract, after a pancreatic RNAase treatment, by isopycnic centrifugation in a CsCl gradient containing ethidium bromide. The [^{3}H]DNA was kept at 4 °C in 15 mM-sodium citrate (pH 7.0)/150 mM-NaCl/1 mM-EDTA (SSCE); its specific radioactivity was 80000 d.p.m./μg.

The ϕX174 RF-I [^{3}H]DNA was depurinated by acid treatment. To 1 vol. of [^{3}H]DNA in SSCE was added 1 vol. of 0.2 M-sodium acetate/acetic acid, pH 3.9, 4.0 being the final pH; after incubation for 90 min at 37 °C, the solution was neutralized with 2 vol. of 1 M-potassium phosphate, pH 12.0. This treatment introduces an average of 1 AP site per RF-I molecule. The depurinated [^{3}H]DNA was dialysed against 50 mM-Epps/KOH (pH 8.0)/1 mM-EDTA; it was kept at 4 °C. When it was utilized, the depurinated RF [^{3}H]DNA contained an average of 0.38 strand interruptions per molecule.

Assay for AP-site excision

[d(-)-1′,2′-^{3}H,5′-^{32}P]DNA was used, the appearance of acid-soluble ^{3}H and ^{32}P being monitored. Nicking on one side of the AP sites releases little acid-soluble radioactivity because the AP site frequency is low (1 AP site/2000 nucleotides). But nicking on both sides of the AP sites releases the totality of the radioactivity in acid-soluble sugar-phosphates.

To the sample (x μl) treated by the nicking agents were added 100 μl of 15 mM-sodium citrate, pH 7.0, 150 mM-NaCl, containing 200 μg of calf-thymus DNA, and $2(100+x)$ μl of 7.5% (w/v) $HClO_4$. After 5 min on ice, the mixture was centrifuged for 10 min at 12000 g. The ^{3}H and ^{32}P radioactivities were measured in a portion of the supernatant; the results are expressed as percentages of the corresponding total radioactivities.

Assays for strand interruptions in ϕX174 RF [^{3}H]DNA

Two methods are used to differentiate between molecules with (RF-II and RF-III) and without (RF-I and RF-IV) strand interruptions: agarose-gel electrophoresis and filtration on nitrocellulose membrane. The average number (n) of strand interruptions per molecule is given by the equation:

$$n = \ln\left[\frac{\Sigma RF}{RF\text{-}I + RF\text{-}IV}\right]$$

Agarose-gel electrophoresis permits the separation of the four RF species (Goffin & Verly, 1982). The DNA samples (20 μl), which had been deproteinized with chloroform/3-methylbutan-1-ol (24/1; v/v), were supplemented with 5 μl of 40% (w/v) sucrose/0.25% Bromophenol Blue before they were placed in the wells of a horizontal 1%-agarose gel in 0.1 M-Tris/borate (pH 8.3)/2 mM-EDTA, containing ethidium bromide (0.5 μg/ml)(TBE-EtB). The electrophoresis was performed for 30 min at 120 V, then for 15 h at 20 V, in TBE-EtB. The bands were revealed under u.v. light, and the gel was cut to separate RF-I + RF-IV from RF-II + RF-III. The gel was melted, and the [^{3}H]DNA was hydrolysed by heating with 1 M-HCl at 100 °C for 15 min before measuring radioactivity.

Filtration on nitrocellulose membrane (Schleicher und Schüll, BA/85), after a cycle of alkaline denaturation

and neutralization, selectively retains the single-stranded DNA deriving from RF-II and RF-III. After deproteinization with chloroform/3-methylbutan-1-ol (24/1, by vol.), the samples were divided into two. After addition of $NaBH_4$ up to 0.4 M to one half, it was incubated at room temperature for 30 min to reduce the AP sites and stabilize those that are still intact. After addition of NaOH up to pH 11 to the other half, it was heated at 65 °C for 60 min in order to nick the DNA strands near all the AP sites. The two halves (25 μl each) were subsequently treated in the same way: they were diluted with 1 ml of potassium phosphate (pH 11.7)/0.9 M-NaCl/20 mM-EDTA and incubated for 10 min at 30 °C; after neutralization with 0.5 ml of 2 M-Tris/HCl (pH 3.2), RF-I and RF-IV returned to their original conformations, whereas RF-II and RF-III remained denatured; the two samples were again diluted with 2 ml of 50 mM-Tris/HCl (pH 8.0)/1 M-NaCl/10 mM-EDTA; they were finally deposited on the nitrocellulose membrane and slowly filtered (5 ml/min); the tubes were rinsed with 5 ml of 90 mM-sodium citrate (pH 7.0)/900 mM-NaCl/6 mM-EDTA, which was also poured on the membrane. The membrane was dissolved in 1 ml of ethyl acetate before measuring the radioactivity.

Nicking reactions

Alkaline treatment. NaOH was added to a final concentration of 0.2 M to [d(-)-1′,2′-^{3}H,5′-^{32}P]DNA samples which were then incubated for 15 min at 37 °C. This treatment is sufficient to cleave the DNA strands at each AP site (Paquette *et al.*, 1972).

Polyamine or histone H1 treatments. Samples of [5′-^{32}P]pdT$_8$d(-)dT$_n$ (1 μM) in 50 mM-Hepes/KOH/1 mM-EDTA, pH 8.0, were incubated at 37 °C for 4 h with histone H1 (3 μM) or for 2 h with spermidine or spermine (1 mM). Analysis by polacrylamide-gel electrophoresis was performed as described by Bailly & Verly (1987).

Samples of [d(-)-1′,2′-^{3}H,5′-^{32}P]DNA (150 μg/ml or 230 μM nucleotide pairs) in 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA, containing 1 mM-polyamine (spermine or spermidine) or 23 μM-histone H1, were incubated at 37 °C for 90 or 240 min respectively.

Samples of partially depurinated φX174 RF-I [^{3}H]DNA (50 μg/ml) in 50 mM-Epps/KOH (pH 8.0)/1 mM-EDTA, containing 0.1 mM-spermine, were incubated 30 min at 37 °C.

AP endonuclease treatments. Samples of [d(-)-1′,2′-^{3}H,5′-^{32}P]DNA (150 μg/ml) in 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA/10 mM-$MgCl_2$, were incubated for 30 min at 37 °C with an excess of chromatin AP endonuclease from rat liver.

Samples of partially depurinated φX174 RF-I [^{3}H]DNA (50 μg/ml) in 50 mM-Epps/KOH (pH 8.0)/1 mM-EDTA/10 mM-$MgCl_2$, were incubated for 10 min at 37 °C with an excess of chromatin AP endonuclease from rat liver.

The AP endonuclease treatment used to show the presence of intact AP sites subsisting or reappearing after the repair steps was performed on a part of the DNA sample which had been heated 5 min at 65 °C to inactivate the enzymes; without changing the medium composition, an excess of AP endonuclease was added and the mixture was incubated for 10 min at 37 °C.

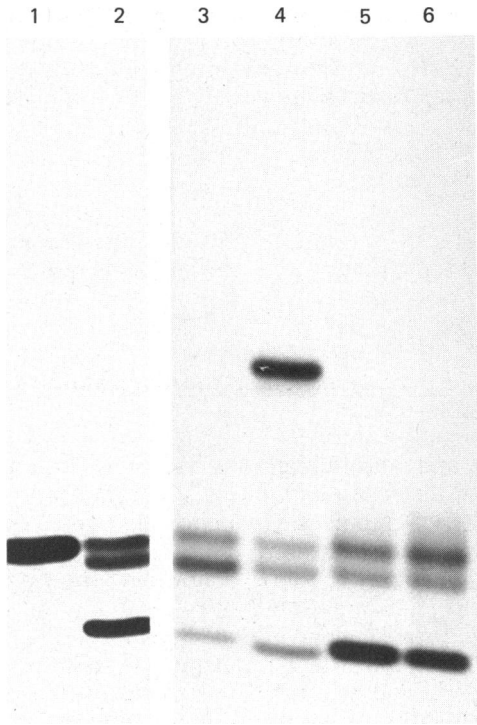

Fig. 1. Histone H1, spermidine and spermine induce β-elimination and βδ-elimination reactions at AP sites in polydeoxynucleotides

[5′-^{32}P]pdT$_8$d(-)dT$_n$ was incubated for 2 h at 37 °C in 0.05 M-NaOH (lane 2). The same substrate was incubated, at pH 8.0 and 37 °C, for 4 h with 3 μM-histone H1 (lane 3) or for 2 h with 1 mM-spermidine (lane 5) or spermine (lane 6). The reaction products were submitted to electrophoresis in a polyacrylamide gel that was subsequently autoradiographed. A reference marker of [5′-^{32}P]pdT$_8$dA was electrophoresed in lane 1. [5′-^{32}P]-pdT$_8$-d(-)dT$_n$ was also incubated at 37 °C for 3 h with 1 mM-Lys-Trp-Lys in 25 mM-sodium cacodylate/1 mM-EDTA, pH 6.5, and the products were analysed by electrophoresis (lane 4). The radioactive band above the β- and βδ-elimination 5′ products is likely the Schiff base formed between the tripeptide and the aldehyde of the 5′ β-elimination product (see also Hélène *et al.*, 1982). The Figure is a composite from two different electrophoretic runs (1–2 and 3–6).

Polymerization and ligation

Samples of φX174 RF [^{3}H]DNA (10 μg/ml) in 50 mM-Epps/KOH (pH 8.0)/0.1 mM-EDTA/10 mM-$MgCl_2$/2 mM-dithiothreitol/nucleotides (40 μM each of dATP, dGTP, dCTP and dTTP)/400 μM-ATP were incubated for 2 h at 15 °C with DNA polymerase-β (0.52 unit/μg of DNA) and T_4 DNA ligase (2.8 units/μg of DNA).

RESULTS

Histone H1, spermine and spermidine catalyse β- and βδ-elimination reactions at AP sites

We have shown previously that, when [5′-^{32}P]-pdT$_8$d(-)dT$_n$ was incubated for 2 h at 37 °C in 0.05 M-NaOH to cleave the sugar-phosphate backbone at the AP sites, three radioactive 5′ fragments were produced that gave three bands after polyacrylamide-gel electro-

Table 1. Excision of AP sites when incubation with AP endonuclease is followed by a treatment with a β-elimination catalyst

[d(-)-1′,2′-^{3}H,5′-^{3}H]DNA was incubated successively with or without chromatin AP endonuclease from rat liver, then with or without a β-elimination catalyst [NaOH, spermidine (spd) or spermine (sp)].

		Acid-soluble radioactivity (% of total substrate radioactivity)					
	AP endonuclease...	−	+	−	+	+	+
Label	β-Elimination catalyst...	−	−	NaOH	NaOH	spd	sp
^{3}H		0	11	29	98	98	99
^{32}P		0	16	6	103	101	100

phoresis and autoradiography (see Fig. 1, lane 2): a doublet at the position of the pdT_8dA reference marker is the result of β-elimination (which leaves two 5′ products), whereas the third band (pdT_8p) is produced by βδ-elimination (Bailly & Verly, 1987, where the structure of the β- and βδ-elimination products can be found).

In the present work, we demonstrate (Fig. 1) that nicking [5′-^{32}P]$pdT_8d(-)dT_n$, at pH 8.0, with histone H1 (lane 3), spermidine (lane 5), or spermine (lane 6) gives the same qualitative results as the alkaline treatment: the β-elimination doublet and a lower band resulting from βδ-elimination.

We have also tried, at pH 6.5, Lys-Trp-Lys, which is known to nick DNA strands near AP sites (Pierre & Laval, 1981; Behmoaras *et al.*, 1981). Fig. 1, lane 4, indicates that the tripeptide also induces a β-elimination that can be followed by δ-elimination.

We thus confirm that the nicking of polydeoxynucleotides at AP sites induced by molecules containing amino groups results from a β-elimination reaction, and that this can be followed by δ-elimination. The βδ-elimination leaves a gap limited by 3′-phosphate and 5′-phosphate.

Excision of AP sites by the conjugated actions of a β-elimination catalyst and the chromatin AP endonuclease of rat liver

Table 1 shows that untreated [d(-)-1′,2′-^{3}H,5′-^{32}P]-DNA did not contain radioactive acid-soluble fragments.

When it was treated with an excess of AP endonuclease, the acid-soluble fraction accounted for 11 and 16% of the ^{3}H and ^{32}P radioactivity respectively. This was due to the fragmentation of the DNA strands resulting from the nicking 5′ to AP sites and some non-specific activity (see below).

When [d(-)-1′,2′-^{3}H,5′-^{32}P]DNA was treated with NaOH, the acid-soluble fraction rose to account for 6% of the ^{32}P label; this was also due to fragmentation of the DNA strands. The lower value with the alkaline treatment (6%) compared with that observed after the AP endonuclease treatment (16%) must likely be explained by a contamination of the enzyme preparation with non-specific nucleases. After the alkaline treatment, the acid-soluble fraction rose to account for 29% of the ^{3}H label. This much higher value (compared with the 6% for ^{32}P) was due to the loss of a tritiated H^+ from the 2′ position of the base-free deoxyribose during the OH^--catalysed β-elimination, but also to some δ-elimination releasing the ^{3}H-labelled 3′-terminal sugar.

When [d(-)-1′,2′-^{3}H,5′-^{32}P]DNA was treated successively with the AP endonuclease and NaOH, all the ^{3}H and ^{32}P radioactivity became associated with the acid-soluble fraction. This indicated that the AP endonuclease hydrolysed the phosphodiester bond 5′ to all AP sites, leaving a 5′-[^{32}P]phosphorylated base-free [^{3}H]deoxyribose, and that the subsequent alkaline treatment completely released this 5′ terminal base-free sugar 5′-phosphate.

Table 1 shows that the same results were obtained when the alkaline treatment was replaced by an incubation, at pH 8.0, with spermine or spermidine. Thus, after the 5′ incision by the AP endonuclease, these β-elimination catalysts can excise the AP site. The double treatment must leave a one-nucleotide gap limited by a 3′-OH and a 5′-phosphate.

Table 2 indicates that histone H1, spermine and spermidine can introduce a nick near an intact AP site; the results are analogous to those obtained by an alkaline treatment and have the same explanation: in all cases there is a β-elimination partially followed by δ-elimination. What is remarkable is that the ^{3}H and ^{32}P acid-soluble radioactivities did not change when the treatment with the polyamino molecules was followed by a treatment with the chromatin AP endonuclease from rat liver. The polyamino molecules do not inhibit the AP endonuclease (results not shown), so that we must conclude that the 5′ β-elimination product is not a substrate for the chromatin AP endonuclease from rat liver. This is most surprising, since it remains a substrate for the AP endonucleases of *E. coli* (endonuclease VI or endonuclease IV) (results not shown).

Repair with DNA polymerase-β and T_4 DNA ligase of one-nucleotide gaps produced by the successive actions, on AP sites, of chromatin AP endonuclease from rat liver and spermine

In the previous section, we have shown that, after nicking 5′ to an AP site with the AP endonuclease from rat-liver chromatin, the 5′-terminal base-free deoxyribose 5′-phosphate could be released by β-elimination catalysts. The two successive reactions must leave a one-nucleotide gap limited by a 3′-OH and a 5′-phosphate group, repairable with DNA polymerase-β and DNA ligase.

Samples of phage φX174 depurinated RF-I [^{3}H]DNA were incubated successively with chromatin AP endonuclease from rat liver, spermine, DNA polymerase-β and T_4 DNA ligase. Controls without one or several enzymes or without spermine were carried out. The average number of strand interruptions per RF molecule

Table 2. Chromatin AP endonuclease from rat liver cannot excise an AP site after 3′ nicking by β-elimination

The experiment is the same as that described in Table 1, except that the order of treatments has been inverted.

		Acid-soluble radioactivity (% of total substrate radioactivity)						
	β-Elimination...	−	Histone H1		Spermidine		Spermine	
Label	AP endonuclease...	−	−	+	−	+	−	+
^{3}H		0	15	18	28	31	33	35
^{32}P		0	6	7	6	6	6	6

Table 3. Repair of φX174 depurinated RF-I DNA

This Table presents the results of two different experiments. Phage φX174 depurinated RF-I DNA was successively treated with chromatin AP endonuclease (AP endo), followed by spermine (expt. I) or with spermine followed by chromatin AP endonuclease (expt. II), then with DNA polymerase-β (DNA pol) and T_4 DNA ligase (DNA lig). Controls were carried out without one or several enzymes or spermine. The number of strand interruptions per RF molecule was calculated from the results of agarose-gel electrophoresis (n_1) or nitrocellulose membrane filtration (n_3). An aliquot of each sample was submitted to an additional treatment with the AP endonuclease before it was electrophoresed on agarose gel (n_2) or to an alkaline treatment before being filtered on nitrocellulose membrane (n_4).

		No. of strand interruptions/RF molecule (n)							
	Column...	0	1	2	3	4	5	6	7
Expt. I	Expt. II								
AP endo...	Spermine...	−	+	−	+	+	−	+	+
Spermine...	AP endo...	−	−	+	+	−	+	+	+
DNA pol...	DNA pol...	−	−	−	−	−	−	−	+
DNA lig...	DNA lig...	−	−	−	−	+	+	+	+
	n_1								
	(I)	0.47	1.60	1.42	1.57	0.69	1.43	1.01	0.59
	(II)	0.46	1.61	1.06	1.60	1.59	0.49	1.57	1.49
Additional AP endo...		+	+	+	+	+	+	+	+
	n_2								
	(I)	1.36	1.66	1.52	1.58	1.35	1.61	1.03	0.66
	(II)	1.40		1.49			1.48		1.50
	n_3								
	(I)	0.39	1.50	1.42	1.40	0.79	1.47	1.17	0.63
	(II)	0.34	1.45	0.92	1.40	1.34	0.46	1.45	1.37
	n_4								
	(I)	1.47	1.55	1.54	1.57	1.34	1.55	1.26	0.65
	(II)	1.47	1.40	1.49	1.44	1.50	1.48	1.44	1.44

was calculated from the fraction of molecules without strand interruptions (RF-I+RF-IV) determined by agarose-gel electrophoresis or nitrocellulose membrane filtration (see the Materials and methods section). To determine whether the RF DNA still contained intact AP sites, it was treated a second time with the AP endonuclease (agarose-gel electrophoresis) or exposed to an alkaline pH (nitrocellulose-membrane filtration).

Table 3 shows the results of this experiment [rows labelled '(I)']. We shall comment only on the agarose-gel-electrophoresis data; the discussion would be the same for that obtained with nitrocellulose-membrane filtration, the two techniques giving very similar results.

When the buffers did not contain any enzyme or spermine (column 0), the RF DNA was left with an average of 0.47 strand interruptions per molecule; this RF DNA contained intact AP sites, since, after a subsequent treatment with the AP endonuclease, this value rose to 1.36.

When incubated with the AP endonuclease (column 1), or with spermine (column 2), or with AP endonuclease followed by spermine (column 3), the RF DNA was quantitatively nicked at the AP sites, since an additional treatment with the AP endonuclease did not significantly modify the number of strand interruptions.

The interruptions made by the AP endonuclease from rat liver chromatin are simple nicks limited by 3′-OH and 5′-phosphate ends (César & Verly, 1983); the absence of a base on the 5′-terminal deoxyribose residue did not prevent T_4 DNA ligase from sealing the nick, since the number of interruptions decreased from 1.60 (column 1) to 0.69 (column 4). The reappearance of intact AP sites was shown by their sensitivity to a second treatment with the AP endonuclease (0.69→1.35).

The strand interruptions made by spermine were not sealed by T_4 DNA ligase (compare the results in columns 2 and 5). This is easy to understand, since 3′ nicking by β-elimination does not leave a 3′-OH end.

Column 7 shows that the one-nucleotide gap produced by the successive actions of AP endonuclease and spermine was filled by DNA polymerase-β, and the continuity of the strand was re-established by T_4 DNA ligase. Indeed the number of strand interruptions decreased from 1.57 (column 3) to 0.59 (column 7). This repaired DNA was practically devoid of intact AP sites, since an additional treatment with the AP endonuclease did not significantly change the number of strand interruptions (0.59→0.66). The DNA had thus been efficiently repaired; this confirms that the one-nucleotide gaps left by the AP endonuclease and spermine were limited by 3′-OH and 5′-phosphate ends.

Surprising results are presented in column 6: the strand interruptions made by the successive actions of AP endonuclease and spermine (one-nucleotide gaps) can be closed by T_4 DNA ligase alone. This was confirmed by using synthetic oligonucleotides (Goffin *et al.*, 1987). The interpretation is that the nucleotide in front of the gap can be excluded from the double helix, allowing the restacking of the flanking nucleotide pairs, which draws close to one another the 3′-OH and 5′-phosphate ends to be ligated. Comparison of the results in columns 3, 6 and 7 shows that T_4 DNA ligase works better when the gaps have been filled by DNA polymerase-β (1.57→0.59 instead of 1.57→1.01).

We also tested another sequence of the repair steps: ϕX174 depurinated RF-1 [^{3}H]DNA was first nicked with spermine, then incubated with the AP endonuclease from rat liver chromatin before trying to complete the repair with DNA polymerase-β and T_4 DNA ligase. The results are in Table 3 [rows labelled (II)]: no repair occurred. This negative result was not unexpected; it is in agreement with the observation reported above that the chromatin AP endonuclease from rat liver is unable to hydrolyse the 5′ phosphodiester bond of an AP site previously nicked on its 3′ side by β-elimination.

DISCUSSION

Spontaneous breakage of DNA strands at AP (apurinic or apyrimidinic) sites is very slow at physiological pH; the average lifetime of an intact AP site (i.e. non-associated with a break) has been calculated to be 190 h at 37 °C (Lindahl & Anderson, 1972). However, the stability of intact AP sites is greatly decreased by basic amino molecules, which are supposed to nick the DNA strands by catalysing a β-elimination, possibly after the formation of a Schiff base. This effect has been reported for Tris and glycine (Tamm *et al.*, 1952), basic proteins (MacDonald & Kaufman, 1954), lysine and putrescine (Lindahl & Anderson, 1972), ribonuclease (Thibodeau & Verly, 1977), the tripeptides Lys-Trp-Lys and Lys-Tyr-Lys (Pierre & Laval, 1981; Behmoaras *et al.*, 1981) and polyamines (Male *et al.*, 1982). In the present study we show that basic molecules like histone H1, spermidine and spermine, or Lys-Trp-Lys, nick DNA strands containing AP sites by β-elimination and that this β-elimination can be followed by δ-elimination.

Thibodeau & Verly (1980) have shown that the AP endonuclease of rat liver was mostly located in chromatin; Bricteux-Grégoire *et al.* (1983) found the enzyme in the cores and the linkers. The hypothesis was then that the AP endonuclease was mobile and continuously prospecting the condensed chromatin. Goffin & Verly (1982, 1984) were able to repair DNA containing AP sites *in vitro* by the successive actions of three enzymes extracted from rat liver chromatin (AP endonuclease, DNAase IV, DNA polymerase-β) and T_4 DNA ligase. To observe the repair, the two enzymes that excised the AP sites (AP endonuclease and DNAase IV) had to be inactivated before addition of DNA polymerase-β and DNA ligase. There was indeed antagonism between the AP endonuclease and DNA ligase, and between the DNAase IV (a 5′→3′ exonuclease) and DNA polymerase-β. Goffin & Verly (1982) had shown that the incision 5′ to the AP site performed by the AP endonuclease was easily closed by T_4 DNA ligase; we confirmed their result in the present study. On the other hand, the 5′→3′ exonuclease widens a gap that DNA polymerase-β tries to fill. These antagonisms do not seem to exist in *E. coli* (Gossard & Verly, 1978): the 3′→5′ exonuclease activity (exonuclease III) of endonuclease VI (the main AP endonuclease of this microorganism) enlarges into a gap the incision made 5′ to the AP site and thus works as an anti-ligase; the 5′→3′ exonuclease and DNA polymerase are two activities of the same protein (DNA polymerase-I) that catalyse the translation of a nick which is easily closed by DNA ligase. The antagonisms, observed in mammalian cells, between enzymes involved in the repair of AP sites raise the question of how the cell proceeds to order the repair steps.

A possible answer appeared when Grondal-Zocchi & Verly (1985) analysed the excision of the AP site by the chromatin DNAase IV from rat liver after the DNA strand had been incised with the AP endonuclease; they found that it was the first phosphodiester bond 3′ to the 5′-terminal AP site which was cut and not the second or the third, as was the case with the 5′→3′ exonuclease of DNA polymerase I (Gossard & Verly, 1978). Moreover, they discovered that the 3′ nicking was the result not of a hydrolysis, but of a β-elimination reaction. We then realized that not a single mammalian enzyme had been convincingly shown to hydrolyse a phosphodiester bond 3′ to an AP site and that, in the chromatin environment extremely rich in amino groups, the nicking on this side of the damage could well be due to a β-elimination cleaving the first 3′ phosphodiester bond; histones and polyamines would play a major role in the repair of AP sites.

Our present hypothesis is that the excision steps of AP site repair are carried out by the chromatin AP endonuclease and a β-elimination catalyst. This removes the antagonism between the 5′→3′ exonuclease and DNA polymerase-β that was considered above, since an exonuclease is no longer required; the excision leaves a one-nucleotide gap, which is more 'economical' than a larger gap produced by an exonuclease. We had then to find the order of the two reactions.

Our first guess was that, the AP endonuclease being immobilized in the condensed chromatin, the repair started by a β-elimination reaction, because this would suppress the second antagonism mentioned above, since we have shown that the 3′ nick cannot be sealed by DNA ligase. The 3′ nick would activate the poly(ADP-ribose) polymerase leading to chromatin decondensation (Fréchette *et al.*, 1985), which would set free the AP

endonuclease to nick 5′ to the AP site. The one-nucleotide gap would then be filled by DNA polymerase-β and DNA ligase. We have shown that such a sequence of steps is not possible when histone H1, spermine or spermidine catalyses the first nicking 3′ to the AP site.

We thus had to consider the other possibility. The repair begins with the AP endonuclease that nicks 5′ to the AP site; the enzyme must thus have some mobility to continuously scan the DNA within the condensed chromatin. The second step is the excision of the AP site by β-elimination. Finally the one-nucleotide gap is closed by DNA polymerase-β and DNA ligase. This scheme does not suppress the competition between AP endonuclease and DNA ligase, but it might not be important if the ligation rate is low compared with the rate of nicking. We have shown experimentally *in vitro* with spermine that AP site repair can be carried out in this way.

It cannot be excluded, however, that 3′ nicking by β-elimination occurs first. Would such a nick be a lethal lesion because the AP endonuclease cannot eliminate the 3′-terminal base-free sugar? The answer is no: we have shown that histone H1 and polyamines can also catalyse a δ-elimination that leaves a gap limited by a 3′-phosphate and a 5′-phosphate group identical with that produced by ionizing radiations (Henner *et al.*, 1982). Such gaps are known to be repaired. Habraken & Verly (1983) have found a chromatin 3′-phosphatase that specifically hydrolyses 3′-phosphates into 3′-OH. Thus the succession of β-elimination, δ-elimination and 3′-phosphate hydrolysis leads to the same result as the succession of hydrolysis by the AP endonuclease and β-elimination: a one-nucleotide gap, limited by 3′-OH and 5′-phosphate, that can be closed by DNA polymerase-β and DNA ligase.

A last remark concerns our observation that filling the one-nucleotide gap with DNA polymerase-β is not an absolute prerequisite to ligation. We do not think, however, that such misrepair, which would give a strand with a one-nucleotide deletion ('frameshift mutation'), often occurs. Ligation is much slower when the missing nucleotide has not been replaced, so that addition of one nucleotide by DNA polymerase-β likely has a much higher probability than ligation across the gap.

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REFERENCES

Axelrod, N. (1976) J. Mol. Biol. **108**, 753–770
Bailly, V. & Verly, W. G. (1984) FEBS Lett. **178**, 223–227
Bailly, V. & Verly, W. G. (1987) Biochem. J. **242**, 565–572
Behmoaras, T., Toulmé, J. J. & Hélène, C. (1981) Nature (London) **292**, 858–859
Bricteux-Grégoire, S., Habraken, Y. & Verly, W. G. (1983) Eur. J. Biochem. **129**, 516–517
César, R. & Verly, W. G. (1983) Eur. J. Biochem. **129**, 509–516
Clements, J. E., Rogers, S. G. & Weiss, B. (1978) J. Biol. Chem. **253**, 2990–2999
Fréchette, A., Huletsky, A., Aubin, R. J., de Murcia, G., Mandel, P., Lod, A., Grondin, G. & Poirier, G. G. (1985) Can. J. Biochem. **63**, 764–773
Goffin, C., Bailly, V. & Verly, W. G. (1987) Nucleic Acids Res. **15**, 8755–8771
Goffin, C. & Verly, W. G. (1982) Eur. J. Biochem. **127**, 619–623
Goffin, C. & Verly, W. G. (1984) Biochem. J. **220**, 133–137
Gossard, F. & Verly, W. G. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. **35**, 1589 (abstr. 1179)
Gossard, F. & Verly, W. G. (1978) Eur. J. Biochem. **82**, 321–332
Grondal-Zocchi, G. & Verly, W. G. (1985) Biochem. J. **225**, 535–542
Habraken, Y. & Verly, W. G. (1983) FEBS Lett. **160**, 46–50
Hélène, C., Toulmé, J. J., Behmoaras, T. & Cazenave, C. (1982) Biochimie **64**, 697–705
Henner, W. D., Grunberg, S. M. & Haseltine, W. A. (1982) J. Biol. Chem. **257**, 11750–11754
Lindahl, T. & Anderson, A. (1972) Biochemistry **11**, 3618–3623
Lindahl, T., Gally, J. A. & Edelman, G. M. (1969) Proc. Natl. Acad. Sci. U.S.A. **62**, 597–603
MacDonald, M. R. & Kaufman, B. P. (1954) J. Histochem. Cytochem. **2**, 387–394
Male, R., Fosse, V. M. & Kleppe, K. (1982) Nucleic Acids Res. **10**, 6305–6318
Mosbaugh, D. W. & Linn, S. (1980) J. Biol. Chem. **255**, 11743–11752
Mosbaugh, D. W. & Linn, S. (1983) J. Biol. Chem. **258**, 108–118
Mosbaugh, D. W. & Meyer, R. R. (1980) J. Biol. Chem. **255**, 10239–10247
Paquette, Y., Crine, P. & Verly, W. G. (1972) Can. J. Biochem. **50**, 1199–1209
Pierre, J. & Laval, P. (1981) J. Biol. Chem. **256**, 10217–10220
Richards, R. G. & Ramsay-Shaw, B. (1982) Anal. Biochem. **121**, 69–82
Richardson, C. C., Schildkraut, C. L., Aposhian, H. V. & Kornberg, A. (1964) J. Biol. Chem. **239**, 222–232
Tamm, C., Shapiro, H. S. & Chargaff, E. (1952) J. Biol. Chem. **199**, 313–327
Thibodeau, L. & Verly, W. G. (1977) J. Biol. Chem. **252**, 3304–3309
Thibodeau, L. & Verly, W. G. (1980) Eur. J. Biochem. **107**, 555–563
Van der Westhuysen, D. R. & Van Holt, C. (1971) FEBS Lett. **14**, 333–337
Verly, W. G. & Paquette, Y. (1973) Can. J. Biochem. **51**, 1003–1009
Verly, W. G., Paquette, Y. & Thibodeau, L. (1973) Nature (London) **244**, 67–69

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