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δ-Elimination in the repair of AP (apurinic/apyrimidinic) sites in DNA

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[5′-^{32}P]pdT$_8$d(-)dT$_7$, containing an AP (apurinic/apyrimidinic) site in the ninth position, and [d(-)-1′,2′-^{3}H, 5′-^{32}P]DNA, containing AP sites labelled with ^{3}H in the 1′ and 2′ positions of the base-free deoxyribose [d(-)] and with ^{32}P 5′ to this deoxyribose, were used to investigate the yields of the β-elimination and δ-elimination reactions catalysed by spermine, and also the yield of hydrolysis, by the 3′-phosphatase activity of T4 polynucleotide kinase, of the 3′-phosphate resulting from the βδ-elimination. Phage-φX174 RF (replicative form)-I DNA containing AP (apurinic) sites has been repaired in five steps: β-elimination, δ-elimination, hydrolysis of 3′-phosphate, DNA polymerization and ligation. Spermine, in one experiment, and *Escherichia coli* formamidopyrimidine:DNA glycosylase, in another experiment, were used to catalyse the first and second steps (β-elimination and δ-elimination). These repair pathways, involving a δ-elimination step, may be operational not only in *E. coli* repairing its DNA containing a formamidopyrimidine lesion, but also in mammalian cells repairing their nuclear DNA containing AP sites.

INTRODUCTION

AP (apurinic/apyrimidinic) sites resulting from the loss of bases are the most frequent lesions appearing in DNA. In *Escherichia coli*, after the endonuclease VI/exonuclease III has hydrolysed the phosphodiester bond 5′ to the AP site and degraded the 5′-fragment, leaving a 3′-OH end, DNA polymerase I, starting from this 3′-OH end, fills the gap, displaces the AP site at the 5′ end of the 3′ fragment, and hydrolyses the second or third phosphodiester bond 3′ to the AP site (Gossard & Verly, 1978).

We have recently underlined that, in mammalian cells, not a single enzyme has been convincingly shown to hydrolyse a phosphodiester bond 3′ to an AP site; we have then hypothesized that the 3′ nicking might result from a β-elimination reaction catalysed by molecules such as histones or polyamines, which are abundant in chromatin. We have been able to repair *in vitro* DNA containing AP sites by the four successive following steps: hydrolysis of the phosphodiester bond 5′ to the AP sites with the AP endonuclease isolated from rat liver chromatin; 3′ nicking by β-elimination catalysed with spermine; filling the one-nucleotide gap with DNA polymerase-β isolated from rat liver chromatin; restoration of the strand continuity with DNA ligase (Bailly & Verly, 1988).

But, if the repair begins with the nicking 3′ to the AP site by β-elimination, the AP endonuclease from rat-liver chromatin is unable to hydrolyse the phosphodiester bond 5′ to the AP site. We thought that the repair could nevertheless be carried out because, after catalysing a β-elimination, histones and polyamines could eliminate the base-free sugar by a δ-elimination reaction, leaving a gap limited by 3′-phosphate and 5′-phosphate ends (Bailly & Verly, 1988) and that the chromatin contains a 3′-phosphatase (Habraken & Verly, 1983) (Scheme 1).

In the present work we have repaired *in vitro* the DNA replicative form (RF) of phage φX174 containing an AP site by using spermine to excise the base-free sugar, a 3′-phosphatase, DNA polymerase-β, and DNA ligase. The δ-elimination seems to be the limiting step in this repair. Because the formamidopyrimidine:DNA glycosylase (FPG) of *E. coli* also nicks the DNA strand by catalysing a β-elimination which is immediately followed by a δ-elimination (Bailly *et al.*, 1989), we have used this enzyme instead of spermine in another experiment. We show that the DNA RF of φX174 containing an AP site can be repaired by the successive actions of FPG, 3′-phosphatase, DNA polymerase-β and DNA ligase.

MATERIALS AND METHODS

[5′-^{32}P]pdT$_8$d(-)dT$_7$

The 5′ end of dT$_8$dGdT$_7$ (Eurogentec) was phosphorylated with [γ-^{32}P]ATP (Amersham International) and T4 polynucleotide kinase (Pharmacia). The labelled oligonucleotide was purified on a NENSORB cartridge (NEN Research Products) and eluted in ethanol/water (1:4, v/v). Depurination of [^{32}P]pdT$_8$dGdT$_7$ into [^{32}P]pdT$_8$d(-)dT$_7$, where the AP site is noted by d(-), was obtained by incubation in 30 mM-HCl for 24 h at 37 °C (Tamm *et al.*, 1952).

DNA containing doubly labelled AP sites

The preparation was described by Bailly & Verly (1987). Doubly labelled dUTP was obtained by deamination of [α-^{32}P]dCTP and [1′,2′,5-^{3}H]dCTP (Amersham International). The deamination mixture, together with dATP, dGTP, dCTP and dTTP, was used for the synthesis of DNA; the dTTP/dUTP ratio was 500:1. The newly synthesized radioactive DNA was

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

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incubated with *E. coli* uracil:DNA glycosylase until the [5-^{3}H]uracil was completely released. The DNA then contained AP sites labelled with ^{3}H in the 1′ and 2′ positions of the base-free deoxyribose and with ^{32}P on the phosphate 5′ to this base-free deoxyribose; it is represented by [d(-)-1′, 2′-^{3}H, 5′-^{32}P]DNA. The specific radioactivities were 37500 d.p.m./μg for ^{3}H, and 24000 d.p.m./μg for ^{32}P; 95% of ^{3}H and of ^{32}P were correctly placed, the remaining 5% being in dCMP residues. From the information given by Amersham International on the [1′,2′,5-^{3}H]dCTP, it is deduced that 40% of the ^{3}H localized at AP sites was in the 1′ position of the base-free deoxyribose and 60% in the hydrogen of the 2′ position which is *cis* relative to the labelled 1′ hydrogen in the sugar cyclic β-form.

The acid-soluble radioactivity, i.e. not precipitable in 5% $HClO_4$ at 0 °C, was measured as described by Bailly & Verly (1987).

ϕX174 depurinated RF-I [^{3}H]DNA

The RF-I of phage ϕX174 DNA labelled with ^{3}H (80000 d.p.m./μg) was prepared from *E. coli* HF4733 infected with phage ϕX174 and cultured in the presence of chloramphenicol in a medium containing [*methyl*-^{3}H]thymidine (Amersham) as described by Axelrod (1976). Several protein extractions and RNAase treatments preceded the last step of centrifugation on a CsCl gradient containing ethidium bromide.

The RF-I [^{3}H]DNA was depurinated by a 90 min incubation at 37 °C and pH 4 as described by Goffin & Verly (1982) to have about 1 AP site per molecule. The depurinated [^{3}H]DNA (40 μg/ml) was conserved at 4 °C in 50 mM-Epps/KOH (pH 8.0)/1 mM-EDTA.

Agarose-gel electrophoresis in the presence of ethidium bromide was used to differentiate between DNA molecules with (RF-II and RF-III) and without (RF-I and RF-IV) strand interruptions. The average number n of strand interruptions per RF molecule is given by the equation:

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

Polyacrylamide-gel electrophoresis and autoradiography

The labelled oligonucleotide samples were diluted at least six-fold with stop solution [90% formamide in 0.1 M-Tris/borate (pH 8.3)/2 mM-EDTA/0.05% Xylene Cyanol/0.05% Bromophenol Blue]. Portions (10 μl) were placed in the wells (12 mm) of a 20%-polyacrylamide denaturing gel of 0.8 mm thickness [19% acrylamide (2X Serva)/1% bisacrylamide (2X Serva)/8.3 M-urea (Serva, analytical grade)/0.1 M-Tris/borate (pH 8.3)/2 mM-EDTA]. Electrophoresis was carried out at 700 V for 15 h. The autoradiography was performed at −70 °C with Fuji X-ray films using a Kodak X-Omatic superfast intensifying screen.

Enzymes and other reagents

T4 polynucleotide kinase was bought from Pharmacia. The rat liver AP endonuclease and the DNA polymerase-β were the same as those used previously (Bailly & Verly, 1988). T4 DNA ligase came from BRL Research Products. Spermine was from Sigma. The FPG was a gift from Dr. J. Laval (Institut Gustave Roussy, Villejuif, France); the glycosylase activity was 396 units/μl (Boiteux *et al.*, 1987).

RESULTS

Action of spermine on [^{32}P]pdT$_8$d(-)dT$_7$

Samples of [^{32}P]pdT$_8$d(-)dT$_7$ (160 pmol of nucleotides), hybridized to poly(dA) (160 pmol of nucleotides) in 5 μl of 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA, containing different concentrations of spermine, were incubated for different times at 37 °C and analysed by electrophoresis on a polyacrylamide gel, which was subsequently autoradiographed (Fig. 1).

A few reference samples were analysed on the same gel: [^{32}P]pdT$_8$dGdT$_7$ (lane 1) and [^{32}P]pdT$_8$d(-)dT$_7$ (lane 4) incubated for 2 h at 37 °C in the same Hepes buffer; [^{32}P]pdT$_8$d(-)dT$_7$ submitted to a mild (lane 2) or strong (lane 3) alkaline treatment. [^{32}P]pdT$_8$d(-)dT$_7$, prepared by depurination of [^{32}P]pdT$_8$dGdT$_7$, migrated somewhat farther than its precursor; the depurination was, however, not complete, since the [^{32}P]pdT$_8$d(-)dT$_7$ preparation still contained a small amount of [^{32}P]pdT$_8$dGdT$_7$ and, moreover, some [^{32}P]pdT$_8$d(-)dT$_7$ was degraded during the electrophoresis (lane 4). The alkaline treatments, mild or strong, completely degraded [^{32}P]pdT$_8$d(-)dT$_7$, but the contaminating [^{32}P]pdT$_8$dGdT$_7$ remained unchanged. Mild alkaline treatment gave three products: two of them, forming a doublet, resulted from the β-elimination reaction (see also Bailly & Verly, 1987, 1988), the third one is [^{32}P]pdT$_8$p formed by a δ-elimination that followed the β-elimination (lane 2). The strong alkaline treatment mainly gave [^{32}P]pdT$_8$p, the $\beta\delta$-elimination product (lane 3).

[^{32}P]pdT$_8$d(-)dT$_7$, hybridized to poly(dA), was incubated for 2 h at 37 °C with 0.1 mM (lane 9), 0.2 mM (lane 10), 0.4 mM (lane 11), 0.6 mM (lane 12), or 1 mM (lane 6)-spermine. In all cases the substrate, [^{32}P]pdT$_8$d(-)dT$_7$, was completely degraded, but the importance of the $\beta\delta$-elimination product increased with the spermine concentration. Other samples were similarly treated, except that the spermine concentration was maintained at 1 mM and the incubation time was varied from 1 to 4 h (lanes 5–8). The amount of $\beta\delta$-elimination product increased with time, whereas the amount of product of only the β-elimination decreased.

From this experiment, it is concluded that the β-elimination reaction was complete even with the mildest spermine treatment, but that, with the longest treatment in the presence of 1 mM-spermine (lane 8), the δ-elimination yield was about 50%.

Action of the 3′-phosphatase activity of T4 polynucleotide kinase on the $\beta\delta$-elimination product (Fig. 1)

[^{32}P]pdT$_8$d(-)dT$_7$ (400 pmol of nucleotides), hybridized to poly(dA) (400 pmol of nucleotides), in 12.5 μl of the previously described Hepes buffer containing 1 mM-spermine, was incubated 6 h at 37 °C. After addition of 12.5 μl of 100 mM-Hepes/KOH (pH 8.0)/2 mM-EDTA/20 mM-$MgCl_2$/4 mM-dithiothreitol, two 9 μl aliquots were taken; T4 polynucleotide kinase [1.8 units (lane 13) or 0.4 unit (lane 14)] was supplemented in 1 μl of solution, and the incubation carried out for another 1 h at 37 °C. Addition of stop solution, electrophoresis and autoradiography were as described above.

The two 3′-phosphatase treatments gave the same results: the [^{32}P]pdT$_8$p, resulting from the δ-elimination has nearly disappeared, giving [^{32}P]pdT$_8$, which forms a band immediately below the β-elimination doublet.

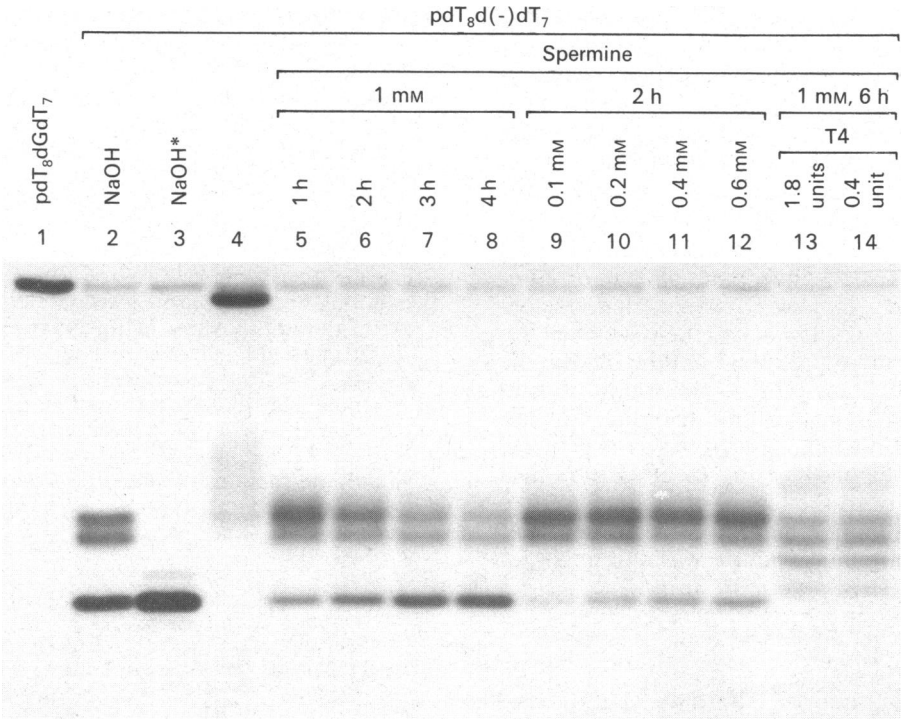


Fig. 1. Actions of spermine and various other treatments on [^{32}P]pdT$_8$d(-)dT$_7$

Lane 1, [^{32}P]pdT$_8$dGdT$_7$; lane 4, [^{32}P]pdT$_8$d(-)dT$_7$. [^{32}P]pdT$_8$d(-)dT$_7$ was submitted to a mild (lane 2) or a strong (lane 3) alkaline treatment. [^{32}P]pdT$_8$d(-)dT$_7$ was hybridized to poly(dA), incubated with 1 mM-spermine at 37 °C for 1 h (lane 5), 2 h (lane 6), 3 h (lane 7) or 4 h (lane 8), or incubated for 2 h at 37 °C with 0.1 mM (lane 9), 0.2 mM (lane 10), 0.4 mM (lane 11) or 0.6 mM (lane 12)-spermine. [^{32}P]pdT$_8$d(-)dT$_7$ was hybridized to poly(dA), treated 6 h at 37 °C with 1 mM-spermine, then incubated with 1.8 units (lane 13) or 0.4 unit (lane 14) of T4 polynucleotide kinase for 1 h at 37 °C. The samples were analysed on a polyacrylamide gel that was afterwards autoradiographed. Abbreviations: NaOH, mild alkaline treatment; NaOH*, strong alkaline treatment; T4, T4 polynucleotide kinase.

Action of spermine on [d(-)-1′,2′-^{3}H, 5′-^{32}P]DNA

After a strong alkaline treatment of [d(-)-1′,2′-^{3}H, 5′-^{32}P]DNA, 4% of the ^{32}P and 95% of the ^{3}H was acid-soluble. The 4% for ^{32}P was due to a complete fragmentation of the DNA, yielding polynucleotides short enough to be acid-soluble; the β-elimination is sufficient for the fragmentation, but because it was always followed by a δ-elimination, the acid-soluble ^{32}P was mostly in the 3′-[^{32}P]phosphate ends of these polynucleotides, the rest being in internal dCMP residues; the acid-soluble ^{32}P was completely adsorbable on Norit. On the other hand, all the ^{3}H labelling in the base-free deoxyribose (95%) was in the acid-soluble fraction. β-Elimination releases as H^+ one of the hydrogen atoms from the 2′ position of the base-free deoxyribose; if the released hydrogen is any one of the two, β-elimination should have set free, in the acid-soluble fraction, 30% of the ^{3}H located at AP sites (see the Materials and methods section), i.e. 28.5% of the total; the subsequent δ-elimination, which sets free an unsaturated derivative of the base-free deoxyribose, should have released the remaining 70%, i.e. 66.5% of the total.

[d(-)-1′, 2′-^{3}H, 5′-^{32}P]DNA (2.6 μg) in 90 μl of 25 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA and 10 μl of spermine solution were incubated at 37 °C. Preliminary experiments showed that the results were largely independent of the spermine concentration over the range of 0.1 to 1 mM. With 0.2 mM-spermine, after a 60 min incubation at 37 °C, the acid-soluble ^{32}P was already at a maximum of 4%, indicating a complete fragmentation of the DNA substrate; we conclude that the β-elimination reaction was already 100% The acid-soluble ^{3}H, which was around 30% after 1 h, rose to 86% after 6 h. This increase was due to the progression of the δ-elimination reaction; one calculates that, after 6 h, the δ-elimination yield was approx. $[(86-28.5)/(95-28.5)] \times 100 = 86\%$. The δ-elimination was thus more complete with this model substrate than with the oligonucleotide used in the previous sections.

To shorten the incubation time, a higher temperature was tested. With 0.2 mM-spermine, after a 3 h incubation at 55 °C, the acid-soluble ^{3}H was 82%, which meant that the δ-elimination yield was about 80%.

Action of the 3′-phosphatase activity of T4 polynucleotide kinase on the products of [d(-)-1′, 2′-^{3}H, 5′-^{32}P]DNA fragmentation by spermine

[d(-)-1′, 2′-^{3}H, 5′-^{32}P]DNA in Hepes buffer, pH 8.0, was incubated with 0.2 mM-spermine for 6 h at 37 °C. To 90 μl of the reaction mixture were added 90 μl of 25 mM-Hepes/KOH (pH 8.0)/2 mM-EDTA/20 mM-$MgCl_2$/4 mM-dithiothreitol; 20 μl aliquots were supplemented with 2 μl of solution containing 0, 0.15, 0.75 or 3.6 units of T4 polynucleotide kinase and incubated 1 h at 37 °C before measuring the Norit-unadsorbable ^{32}P, i.e. the released [^{32}P]P_i. The latter increased with the

amount of 3′-phosphatase to reach 42% of the total ^{32}P when 3.6 units of the enzyme were used. The release of $[^{32}P]P_i$ by the 3′-phosphatase confirms that spermine catalysed a $\beta\delta$-elimination. But the 3′-phosphatase reaction was still incomplete: the $\beta\delta$-elimination catalysed by spermine exposed about 82% (95 × 0.86) of the total ^{32}P in 3′-[^{32}P]phosphate ends (see the preceding section); the highest yield of hydrolysis was thus approx. 50%.

Here again we see a difference between the two substrates: whereas the 3′-phosphate of the $\beta\delta$-elimination product derived from the 5′-labelled oligonucleotide was easily hydrolysed, it was not the case when the starting substrate was [d(-)-1′, 2′-^{3}H, 5′-^{32}P]DNA. But the topology was also different: the 3′-phosphate derived from the oligonucleotide was at the end of an oligomer without anything to hinder the approach of the enzyme, whereas the 3′-phosphate appearing in DNA was in a narrow gap resulting from the loss of a unique base-free sugar. The access was more difficult, but our experiment shows that the 3′-phosphatase action was still possible.

Repair, by DNA polymerase-β and T4 DNA ligase, of a one-nucleotide gap limited by 3′-OH and 5′-phosphate ends in ϕX174 RF [^{3}H]DNA

ϕX174 partially depurinated RF [^{3}H]DNA (containing an average of 1.1 intact AP sites and 0.1 breaks per molecule; 0.4 μg) in 15 μl of 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA/10 mM-$MgCl_2$ and rat liver AP endonuclease in 1 μl of 20 mM-Tris/HCl (pH 8.0)/1 mM-EDTA/0.2 mM-dithiothreitol/10% (v/v) glycerol were incubated 10 min at 37 °C, then heated for 5 min at 65 °C to inactivate the enzyme. To each of two 8 μl portions of the preceding solution were added 2 μl of 50 mM-Epps/KOH (pH 8.0)/1 mM-EDTA/1 mM-spermine, and the mixture was incubated for 1 h at 37 °C. It was subsequently supplemented with 8 μl of DNA polymerase-β solution, 1 μl of T4 DNA ligase solution, 8 μl of the polymerization–ligation mixture and 13 μl of a buffer so that the final concentrations were: 40 μM for each of the four nucleotides (dATP, dGTP, dTTP and dCTP), 400 μM-ATP, 10 mM-$MgCl_2$, 2 mM-dithiothreitol, 50 mM-Epps/KOH, pH 8.0; the incubation was carried out for 2 h at 12 °C. Controls without enzyme or spermine were run in parallel. One of each two identical samples was submitted to a second treatment with rat liver AP endonuclease. Aliquots of 7.5 μl were taken, to which 8.5 μl of water and 4 μl of 40% sucrose/0.25% Bromophenol Blue were added; they were analysed on 1%-agarose gels containing ethidium bromide. The average number of interruptions per RF molecule was calculated as described in the Materials and methods section. The results are shown in Table 1.

The control incubated without enzyme and without spermine contained 0.2 strand interruptions per molecule (column 0); 1.2 − 0.2 = 1.0 intact AP sites were thus left. The AP endonuclease preparation was shown to have no action on intact DNA strands; the first AP endonuclease treatment (column 1) has nicked the strands near 0.84 (1.04 − 0.20) of the 1.00 intact AP sites that would have remained intact in absence of the enzyme; the yield of this first repair step is thus 84%. The spermine treatment (column 2), proceeding by β-elimination, did not leave any intact AP site; the yield of this second repair step is thus 100%. At this stage, the RF DNA contained one-nucleotide gaps limited by 3′-OH and 5′-phosphate ends. After the incubation with the DNA polymerase-β and T4 DNA ligase (column 4), the average number of strand interruptions per molecule decreased from 1.19 to 0.31, and the repaired DNA did not contain reconstituted intact AP sites, as was shown by a second treatment with the AP endonuclease; thus of the 1.0 intact AP sites found when the incubations were carried out without enzyme or spermine, only 0.11 (0.31 − 0.20) were not repaired, which means that the overall yield of repair was: [(1.00 − 0.11)/1.00] × 100 = 89%.

Table 1. Repair of ϕX174 RF [^{3}H]DNA containing a one-nucleotide gap limited by 3′-OH and 5′-phosphate ends

ϕX174 RF [^{3}H]DNA, partially depurinated, was incubated with rat liver AP endonuclease (AP endo). After inactivation of the enzyme, it was incubated with 0.1 mM-spermine for 1 h at 37 °C. Addition of DNA polymerase (DNA pol) and T4 DNA ligase (DNA lig), and the necessary precursors and cofactors, was followed by a 2 h incubation at 12 °C. Controls were carried out without enzyme or spermine. The samples were analysed by electrophoresis on agarose gels containing ethidium bromide. The radioactivities of the RF-I + RF-IV molecules, on the one hand, and of the RF-II + RF-III molecules, on the other hand, were measured and the average number (n) of strand interruptions per molecule was calculated. A part of each sample was submitted to a second treatment with the AP endonuclease and the average number (n') of strand interruptions per molecule was also determined.

Column...	0	1	2	3	4
AP endo...	−	+	+	+	+
Spermine...	−	−	+	+	+
DNA pol...	−	−	−	−	+
DNA lig...	−	−	−	+	+
n	0.20	1.04	1.19	0.75	0.31
AP endo...	+	+	+	+	+
n'	0.98	1.50	1.26	0.72	0.32

Since the overall yield is the product of the yields of the four repair steps, and since the yields of the first and second steps, creating the one-nucleotide gap limited by 3′-OH and 5′-phosphate, were 84% and 100% respectively, the yield of filling the gap with DNA polymerase-β and the yield of re-establishing the strand continuity with T4 DNA ligase were both 100% under the conditions that we used.

Column 3 shows that filling the one-nucleotide gap is not a prerequisite for the action of T4 DNA ligase, confirming results previously published (Goffin *et al.*, 1987; Bailly & Verly, 1988). Ligation is, however, easier when the gap has been filled.

Repair of AP sites in ϕX174 RF [^{3}H]DNA using spermine and a 3′-phosphatase

ϕX174 partially depurinated RF [^{3}H]DNA (containing an average of 1.5 intact AP sites and 0.2 breaks per molecule; 0.4 μg) in 20 μl of 50 mM-Hepes/KOH (pH 8.0)/1 mM-EDTA/0.1 mM-spermine in the same buffer, were incubated 3 h at 55 °C. After addition of 1 μl of T4 polynucleotide kinase solution and 4 μl of 12.5 mM-ATP/62.5 mM-$MgCl_2$/12.5 mM-dithiothreitol, the mixture was incubated 1 h at 37 °C, then heated 5 min

Table 2. Repair of φX174 RF [^{3}H]DNA containing an AP site with spermine, 3′-phosphatase (T4 kinase), DNA polymerase (DNA pol) and DNA ligase (DNA lig)

φX174 RF [^{3}H]DNA, partially depurinated, was incubated with 0.2 mM-spermine for 3 h at 55 °C, with T4 polynucleotide kinase for 1 h at 37 °C, and with DNA polymerase-β and T4 DNA ligase for 2 h at 12 °C. Controls were carried out without spermine and/or enzymes. The number (*n*) of strand interruptions per molecule was determined in each case. Column 00 refers to the unincubated substrate.

Column...	00	0	1	2	3	4	5	6	7	8	9	10
Spermine...	0	−	+	−	−	−	−	+	+	+	+	+
T4 kinase...	0	−	−	−	−	+	+	+	−	−	+	+
DNA pol...	0	−	−	−	+	−	+	−	−	+	−	+
DNA lig...	0	−	−	+	+	+	+	−	+	+	+	+
n	0.21	0.54	1.64	0.53	0.52	0.48	0.43	1.60	1.70	1.67	1.05	0.81

at 65 °C to inactivate the enzyme. Polymerization and ligation steps were carried out as described in the previous section. The samples were analysed in the same way, and the results are presented in Table 2.

Without incubation, the RF [^{3}H]DNA contained an average of 0.21 strand interruptions per molecule (Table 2, column 00), but this value rose to 0.54 in the control where the repair steps were mimicked without enzyme and without spermine (column 0); the difference was mostly due to the 3 h incubation at 55 °C. The treatment with spermine (column 1) nicked the DNA strands near each AP site; an additional incubation with T4 polynucleotide kinase (a 3′-phosphatase) did not change the number of strand interruptions (column 6).

The gaps produced in the depurinated [^{3}H]DNA by spermine were not closed by T4 DNA ligase (column 7) or by the combined actions of DNA polymerase-β and T4 DNA ligase (column 8). However, if the treatment with spermine was followed by a 3′-phosphatase treatment, a subsequent incubation with DNA polymerase-β and T4 DNA ligase decreased the number of strand interruptions per molecule from 1.60 to 0.81 (column 10). Even without DNA polymerase-β, this number was decreased to 1.05 (column 9).

This experiment shows that spermine can excise the base-free sugar by a βδ-elimination, leaving a 3′-phosphate that is hydrolysed by the 3′-phosphatase activity of T4 polynucleotide kinase. The three successive steps leave a one-nucleotide gap that DNA polymerase-β and T4 DNA ligase can fill and close. We shall try to calculate the repair yield. The substrate φX174 RF [^{3}H]DNA contained 1.7 intact AP sites and strand interruptions per molecule. The control treatment without spermine and without enzyme left 1.16 intact AP sites (1.70 − 0.54). The repair left 0.81 strand interruptions, from which must be deduced 0.43 observed when there was no spermine treatment. Thus, of the 1.16 intact AP sites that would have been found if no spermine were used to initiate the repair, 0.38 have not been repaired (0.81 − 0.43). The overall repair yield was thus: $[(1.16-0.38)/1.16] \times 100 = 67\%$.

The repair proceeded in five steps: the yields of the first (β-elimination), the fourth and the fifth (DNA polymerase-β and T4 DNA ligase, explored in the previous section) steps were 100%. Thus the product of the yields of the second (δ-elimination) and the third (3′-phosphatase) was 67%. These values must, of course, be taken as only rough estimates.

Repair of AP sites in φX174 RF [^{3}H]DNA using FPG and a 3′-phosphatase

We have shown (Bailly *et al.*, 1989) that *E. coli* FPG nicks DNA strands near AP sites by catalysing a β-elimination that is immediately followed by a δ-elimination. The experiment was carried out as described in the previous section, except that the spermine treatment was replaced by an incubation with FPG in 50 mM-Hepes/KOH (pH 7.5)/0.1 M-KCl/1 mM-EDTA (3.2 units/μg of DNA). The results are presented in Table 3. One can see that most of the 2.19 strand interruptions per molecule observed after the successive treatments with FPG and T4 polynucleotide kinase (column 2) were closed by the successive actions of DNA polymerase-β and T4 DNA ligase (column 3).

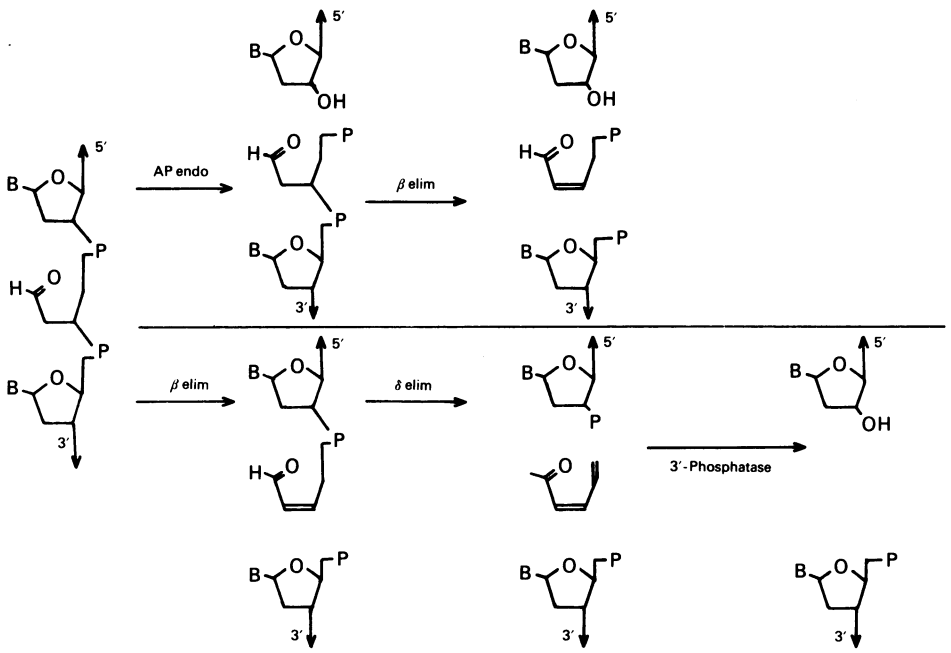
DISCUSSION

In this work, we have investigated the possibility of repairing DNA-containing AP sites by following a five-

Table 3. Repair of φX174 RF [^{3}H]DNA containing an AP site with *E. coli* FPG, 3′-phosphatase (T4 kinase), DNA polymerase (DNA pol) and DNA ligase (DNA lig)

φX174 RF [^{3}H]DNA, partially depurinated, was incubated with *E. coli* FPG for 10 min at 37 °C, with T4 polynucleotide kinase for 1 h at 37 °C, and with DNA polymerase-β and T4 DNA ligase for 2 h at 12 °C. Controls were carried out without one or several enzymes. The number (*n*) of strand interruptions per molecule was determined; results of duplicate electrophoreses indicate the precision of the technique. Column 00 refers to the unincubated substrate.

Column...	00	0	1	2	3
FPG...	0	−	+	+	+
T4 kinase...	0	−	−	+	+
DNA pol...	0	−	−	−	+
DNA lig...	0	−	−	−	+
n	0.35	0.54	1.85	2.19	0.76
	0.36	0.54	1.86	2.13	0.76



Scheme 1. The two proposed pathways of AP-site excision in mammalian cells

The main pathway (above) begins with the hydrolysis of the C3'-O-P bond 5' to the AP site by the chromatin AP endonuclease (AP endo), followed by a β-elimination (β elim) reaction catalysed by histones or polyamines that leaves a one-nucleotide gap limited by 3'-OH and 5'-phosphate ends. The rescue pathway (below) begins with the cleavage of the C3'-O-P bond 3' to the AP site by a β-elimination reaction; the chromatin AP endonuclease is then unable to hydrolyse the C3'-O-P 5' to the AP site, but histones and polyamines that have catalysed the first step of the excision can also catalyse a δ-elimination that releases the base-free sugar, leaving a gap limited by 3'-phosphate and 5'-phosphate ends; the chromatin 3'-phosphatase then hydrolyses the 3'-phosphate into 3'-OH, yielding the same one-nucleotide gap as in the main excision pathway. This gap can be filled with DNA polymerase-β and DNA ligase to complete the repair.

step pathway: β-elimination, δ-elimination, hydrolysis of the 3'-phosphate resulting from the δ-elimination, addition of one nucleotide and final ligation. For this demonstration we used ϕX174 RF DNA labelled with ^{3}H and containing about 1 AP site per molecule; electrophoretic separation of RF-I + RF-IV (with uninterrupted strands) from RF-II + RF-III (containing at least one strand interruption) enabled us to monitor the average number of strand interruptions per molecule.

In a first series of experiments, we used spermine to catalyse the two first repair steps, β-elimination and δ-elimination.

The overall repair yield is the product of the yields of the five successive steps; to observe a detectable repair, it was thus necessary to have, for each step, a sufficiently high yield. But, with ϕX174 RF [^{3}H]DNA, we could only investigate the yield of the first step (β-elimination that converts RF-I into RF-II) and the aggregate fourth and fifth steps (polymerization and ligation that transform RF-II into RF-IV). The nicking near the AP site by β-elimination is easily obtained with spermine; under all the conditions that were explored, the yield was 100%. To measure the combined yields of the fourth and fifth steps, ϕX174 RF [^{3}H]DNA containing a one-nucleotide gap limited by 3'-OH and 5'-phosphate ends was prepared with rat liver AP endonuclease and spermine; under the chosen conditions, the yields of the polymerization and ligation steps were also close to 100%.

But the slower steps were the second (δ-elimination) and the third (hydrolysis of the 3'-phosphate) and we could not use ϕX174 RF [^{3}H]DNA to find good conditions; the [^{3}H]DNA remains indeed in the RF-II form during these second and third steps of repair. It is the reason why we studied these two steps on two other substrates: the oligonucleotide [^{32}P]pdT$_8$d(-)dT$_7$, using gel electrophoresis and autoradiography to monitor its changes; [d(-)-1',2'-^{3}H, 5'-^{32}P]DNA, i.e. a DNA containing AP sites labelled with ^{3}H in the 1' and 2' positions of the base-free deoxyribose, and with ^{32}P in the 5' phosphodiester bond. With this latter substrate, the release of acid-soluble ^{3}H enabled us to monitor the β- and δ-elimination reactions, whereas the hydrolysis of the 3'-phosphate resulting from the δ-elimination could be monitored by the release of Norit-unadsorbable ^{32}P. The two substrates behaved differently.

The δ-elimination yield was largely independent of the spermine concentration; to prevent aggregation of the substrate molecules, we finally chose a rather low concentration (0.2 mM). For similar treatment conditions, the δ-elimination catalysed by spermine had a higher yield with [d(-)-1',2'-^{3}H, 5'-^{32}P]DNA (86%) than with the oligonucleotide (about 50%). We thought that ϕX174 RF [^{3}H]DNA would rather follow the [d(-)-1',2'-^{3}H, 5'-^{32}P]DNA model and that the finally chosen conditions were sufficient (3 h at 55 °C which was equivalent to 6 h at 37 °C).

The same model substrates were used to study the yield of hydrolysis of the 3'-phosphate produced by $\beta\delta$-elimination. We found that the 3'-phosphatase of T4 polynucleotide kinase hydrolysed more easily [^{32}P]pdT$_8$p into [^{32}P]pdT$_8$ than the 3'-[^{32}P]phosphate limiting the gap produced in [d(-)-1',2'-^{3}H, 5'-^{32}P]DNA by $\beta\delta$-elim-

ination. The access is likely easier for the enzyme when the 3′-phosphate is at the end of an oligonucleotide than when it is in a narrow gap within a DNA strand. Nevertheless, this hydrolysis was not impossible, so that we decided to attempt the repair of an AP site within φX174 RF [^{3}H]DNA.

Thus φX174 RF [^{3}H]DNA containing an average of about 1 AP site per molecule was successively treated with spermine, the 3′-phosphatase activity of T4 polynucleotide kinase, DNA polymerase-β and T4 DNA ligase. The first repair step (β-elimination) changed the RF-I [^{3}H]DNA into RF-II; the [^{3}H]DNA remained RF-II during the second, third and fourth step of the repair, to be restored into RF-IV during the fifth step (ligation). Under the chosen conditions, the overall repair yield was about 67%; since the yields of the first, fourth and fifth steps were estimated at 100%, this meant that the product of the yields of the second (δ-elimination) and third (hydrolysis of the 3′-phosphate) steps was about 67%.

In conclusion, δ-elimination can be a step in a pathway to repair DNA containing AP sites. Thus when, in chromatin, histones or polyamines nick the DNA strand 3′ to an AP site by β-elimination and the AP endonuclease can no more hydrolyse the phosphodiester bond 5′ to the AP site (Bailly & Verly, 1988), the repair is still possible: histones or polyamines catalyse a δ-elimination, producing a gap limited by 3′-phosphate and 5′-phosphate, identical with that formed by ionizing radiations (Henner *et al.*, 1982), which is reparable. Indeed chromatin has a 3′-phosphatase (Habraken & Verly, 1983) that hydrolyses the 3′-phosphate into 3′-OH so that, finally, the same one-nucleotide gap is obtained as after the successive actions of AP endonuclease and a β-elimination catalyst. It is highly probable that δ-elimination operates in one of the pathways functioning in mammalian cells to repair nuclear DNA containing AP sites (Scheme 1).

We have seen that, with spermine, the β-elimination proceeds quite rapidly, but that δ-elimination is a slow reaction. *E. coli* FPG is an enzyme that nicks on both sides of AP sites by catalysing a β-elimination immediately followed by δ-elimination (Bailly *et al.*, 1989). In an experiment independent from the previous ones, we successfully repaired φX174 RF [^{3}H]DNA containing an AP site by the successive actions of FPG, the 3′-phosphatase activity of T4 polynucleotide kinase, DNA polymerase-β and T4 DNA ligase. A similar pathway must be followed in *E. coli* to repair its DNA when it contains a formamidopyrimidine derived from a 7-alkylguanine: the glycosylase activity removes the altered base, leaving an AP site, and the nicking activity removes the base-free sugar; the 3′-phosphatase activity of exonuclease III or endonuclease IV hydrolyses the 3′-phosphate into 3′-OH; the repair can then be terminated by DNA polymerase I and DNA ligase.

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