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The multiple activities of *Escherichia coli* endonuclease IV and the extreme lability of 5′-terminal base-free deoxyribose 5-phosphates

Véronique BAILLY and Walter G. VERLY
Biochimie, Faculté des Sciences, Université de Liège, Sart Tilman B6, B-4000 Liège 1, Belgium

Escherichia coli endonuclease IV hydrolyses the $C_{(3')}$-O–P bond 5′ to a 3′-terminal base-free deoxyribose. It also hydrolyses the $C_{(3')}$-O–P bond 5′ to a 3′-terminal base-free 2′,3′-unsaturated sugar produced by nicking 3′ to an AP (apurinic or apyrimidinic) site by β-elimination; this explains why the unproductive end produced by β-elimination is converted by the enzyme into a 3′-OH end able to prime DNA synthesis. The action of *E. coli* endonuclease IV on an internal AP site is more complex: in a first step the $C_{(3')}$-O–P bond 5′ to the AP site is hydrolysed, but in a second step the 5′-terminal base-free deoxyribose 5′-phosphate is lost. This loss is due to a spontaneous β-elimination reaction in which the enzyme plays no role. The extreme lability of the $C_{(3')}$-O–P bond 3′ to a 5′-terminal AP site contrasts with the relative stability of the same bond 3′ to an internal AP site; in the absence of β-elimination catalysts, at 37 °C the half-life of the former is about 2 h and that of the latter 200 h. The extreme lability of a 5′-terminal AP site means that, after nicking 5′ to an AP site with an AP endonuclease, in principle no 5′→3′ exonuclease is needed to excise the AP site: it falls off spontaneously. We have repaired DNA containing AP sites with an AP endonuclease (*E. coli* endonuclease IV or the chromatin AP endonuclease from rat liver), a DNA polymerase devoid of 5′→3′ exonuclease activity (Klenow polymerase or rat liver DNA polymerase β) and a DNA ligase. Catalysts of β-elimination, such as spermine, can drastically shorten the already brief half-life of a 5′-terminal AP site; it is what very probably happens in the chromatin of eukaryotic cells. *E. coli* endonuclease IV also probably participates in the repair of strand breaks produced by ionizing radiations: as *E. coli* endonuclease VI/exonuclease III, it is a 3′-phosphoglycollatase and also a 3′-phosphatase. The 3′-phosphatase activity of *E. coli* endonuclease VI/exonuclease III and *E. coli* endonuclease IV can also be useful when the AP site has been excised by a βδ-elimination reaction.

INTRODUCTION

Endonuclease IV is one of the two known AP endonucleases of *Escherichia coli*; it is responsible for about 10% of the AP endonuclease activity of the bacterial crude extract (Ljungquist *et al.*, 1976; Ljungquist, 1977). The main enzyme, which accounts for about 90% of the total activity, is endonuclease VI (Verly & Paquette, 1970, 1972; Gossard & Verly, 1978); it is the same protein as exonuclease III (Weiss, 1976; Ljungquist & Lindahl, 1977; Gossard & Verly, 1978).

Endonuclease VI has been shown to hydrolyse the phosphodiester bond 5′ to the AP sites, leaving 3′-OH and 5′-phosphate ends (Gossard & Verly, 1976). Endonuclease IV is believed to catalyse the same reaction although, to our knowledge, only indirect arguments have been presented, such as production of a 3′-OH that can be used as primer for *E. coli* DNA polymerase I (Mosbaugh & Linn, 1982) and the position of the nick in a 5′-labelled oligonucleotide, containing a base-less tetrahydrofuran, exposed to the enzyme (Takeshita *et al.*, 1987). In the present paper we show that *E. coli* endonuclease IV indeed hydrolyses the $C_{(3')}$-O–P bond 5′ to the AP sites, but we were surprised to observe that this reaction was followed by the loss of the 5′-terminal base-free deoxyribose 5′-phosphate. We also show that endonuclease IV releases 3′-terminal base-free deoxyribose 5′-phosphate or the 3′-terminal base-free 2′,3′-unsaturated sugar 5′-phosphate resulting from the nicking 3′ to an AP site by β-elimination.

Endonuclease IV was shown to hydrolyse 3′-phosphoglycollate ends into 3′-OH (Demple *et al.*, 1986), and we have confirmed this observation (Siwek *et al.*, 1988); we show here that, in addition, *E. coli* endonuclease IV is also a 3′-phosphatase, confirming the results published by Levin *et al.* (1988).

MATERIALS AND METHODS

Preparation or purification of enzymes

E. coli endonuclease III was prepared as described by Bailly & Verly (1987).

Snake-venom phosphodiesterase (Sigma type IV) was purified on Blue Sepharose (Oka *et al.*, 1978).

E. coli endonuclease IV was purified by the method of Ljungquist (1977) from *E. coli* B41 cells induced with paraquat (Chan & Weiss, 1987); the preparation was kept at −20 °C in 10 mM-Tris/HCl buffer, pH 8.0, containing 0.5 mM-EDTA, 0.5 mM-dithiothreitol, 0.15 M-NaCl and 50% (v/v) glycerol. The protein concentration was 80 μg/ml and the enzyme activity 20 units/ml. The

Abbreviations used: AP, apurinic or apyrimidinic; d(–), symbol for base-free deoxyribose (AP site); s(=), symbol for unsaturated sugar.

endonuclease IV unit is the amount of enzyme producing, in 10 min at 37 °C, 1 pmol of nicks near AP sites in [d(–),5′-^{32}P]DNA dissolved (160 μg/ml) in 50 mM-Hepes/KOH buffer, pH 8.2, containing 1 mM-EDTA, 0.2 M-NaCl, 1 mM-dithiothreitol and bovine serum albumin (0.1 mg/ml); to determine the amount of nicks produced by endonuclease IV, the incubation medium was submitted to a strong alkaline treatment before measurement of the Norit-non-adsorbable ^{32}P.

The preparation of chromatin AP endonuclease from rat liver was that used by Bailly & Verly (1988).

Substrates

Labelled DNAs. DNA singly labelled with ^{32}P 5′ to AP sites, represented by [d(–),5′-^{32}P]DNA, or doubly labelled with ^{32}P 5′ to AP sites and with ^{3}H in the 1′ and 2′ positions of the base-free deoxyribose, represented by [d(–),1′,2′-^{3}H,5′-^{32}P]DNA, were prepared as described by Bailly & Verly (1987).

Labelled oligonucleotides. 5′-Labelling: 60 mM-Tris/HCl buffer, pH 8.0, containing 9 mM-$MgCl_2$, 0.1 M-KCl and 15 mM-2-mercaptoethanol (100 μl), also containing 10 nmol of $dGdT_{11}$, dT_8dGdT_7 (Eurogentec), dT_8dA (Pharmacia) or dT_{8-11}, 15 nmol of [γ-^{32}P]ATP (100 μCi; Amersham) and 10 units of phage-T4 polynucleotide kinase (Pharmacia), was incubated for 1 h at 37 °C.

3′-Labelling: 0.1 M-potassium cacodylate buffer, pH 7.2, containing 2 mM-$CoCl_2$ and 0.2 mM-dithiothreitol (100 μl), also containing 10 nmol of $dT_{11}d(-)dT_{11}$, 15 nmol of [α-^{32}P]ddATP (100 μCi; Amersham) and 70 units of calf thymus deoxynucleotidyl-terminal transferase (Pharmacia), was incubated for 2 h at 37 °C. The 3′-labelled oligonucleotide is called $dT_{11}d(-)dT_{11}$[^{32}P]ddA.

Purification: the oligonucleotides, labelled at the 3′-end or 5′-end, were purified on a NENSORB cartridge (NEN Research Products), and retrieved in ethanol/water (1:4, v/v).

Depurination: [^{32}P]pdT_8dA was depurinated by a 1 h heating at 65 °C in 10 mM-HCl; $dT_{11}dGdT_{11}$ (Eurogentec), [^{32}P]pdT_8dGdT_7 and [^{32}P]$pdGdT_{11}$ were depurinated by a 24 h incubation at 37 °C in 30 mM-HCl; to obtain dT_{8-11}, $dGdT_{11}$ (Eurogentec) was depurinated, submitted to a mild alkaline treatment, dephosphorylated with calf intestine phosphatase (Sigma type VIIS) and slightly degraded by an exonuclease contaminating the phosphatase.

Partially depurinated bacteriophage-ϕX174 RF-1 [^{3}H]DNA. The replicative form 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 [*Me*-^{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-1 [^{3}H]DNA was partially depurinated by a 90 min incubation at 37 °C and pH 4 as described by Goffin & Verly (1982) to contain about 1 AP site per molecule. The partially depurinated [^{3}H]DNA (40 μg/ml) was conserved at 4 °C in 50 mM-Hepps/KOH buffer, pH 8.0, containing 1 mM-EDTA.

Chemical and enzymic reactions

Alkaline treatments. Mild alkaline treatment was in 0.2 M-NaOH for 15 min at 37 °C; strong alkaline treatment was in 0.2 M-NaOH for 30 min at 65 °C.

Spermine. A 10 μl volume of 50 mM-Hepes/KOH buffer, pH 8.0, containing 1 mM-EDTA and 0.2 mM-spermine, also containing [^{32}P]$pd(-)dT_{11}$ (240 pmol of nucleotides) and poly(dA) (480 pmol of nucleotides), was incubated for 10 min at 37 °C.

***E. coli* endonuclease VI/exonuclease III.** A 10 μl volume of 50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$ and 1 mM-2-mercaptoethanol, also containing [^{32}P]$pdT_8d(-)dT_7$ (320 pmol of nucleotides), poly(dA) (640 pmol of nucleotides) and 6.5 units of *E. coli* exonuclease III (Bethesda Research Laboratories), was incubated for 15 min at 16 °C.

Snake-venom phosphodiesterase. A 10 μl volume of 50 mM-Hepes/KOH buffer, pH 8.0, containing 10 mM-$MgCl_2$, 1 mM-2-mercaptoethanol, 2 mM-ATP, also containing [^{32}P]$pdT_8d(-)dT_7$ and 33 μunits of snake-venom phosphodiesterase, was incubated for 10 min at 37 °C.

***E. coli* endonuclease IV.** A 10 μl volume of 50 mM-Hepes/KOH buffer, pH 8.2, containing 1 mM-EDTA, 0.2 M-NaCl and 1 mM-dithiothreitol, also containing 20 pmol of the radioactive oligonucleotide, poly(dA) (twice the nucleotide-equivalent amount) and *E. coli* endonuclease IV, was incubated for 2 h at the indicated temperature.

Also, a 950 μl volume of 50 mM-Hepes/KOH buffer, pH 8.2, containing 1 mM-EDTA, 0.2 M-NaCl, 1 mM-dithiothreitol and 0.1 mg of bovine serum albumin/ml also containing 100 μg of [d(–),5′-^{32}P]DNA or [d(–),-1′,2′-^{3}H,5′-^{32}P] DNA and 50 μl of *E. coli* endonuclease IV preparation (1 unit), was incubated at 37 °C.

***E. coli* endonuclease III.** A 10 μl volume of 50 mM-Hepes/KOH buffer, pH 7.8, containing 0.1 M-KCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol, also containing [^{32}P]$pdT_8d(-)dT_7$ (320 pmol of nucleotides), poly(dA) (640 pmol of nucleotides) and *E. coli* endonuclease III, was incubated for 2 h at 26 °C.

Calf spleen phosphodiesterase. A 10 μl volume of 10 mM-imidazole/HCl buffer, pH 6.0, containing 0.1 mM-EDTA and 25 mM-methoxyamine, also containing 80 pmol of $dT_{11}d(-)dT_{11}$[^{32}P]ddA and the indicated amount of calf spleen phosphodiesterase (Merck), was incubated for 10 min at 37 °C; the enzyme was then inactivated by a 5 min heating at 65 °C.

Bacteriophage-T4 polynucleotide kinase. To one-half of the sample treated with calf spleen phosphodiesterase were added 4 μl of 150 mM-Hepes/KOH buffer, pH 8.0, containing 22.5 mM-$MgCl_2$, 0.25 M-KCl, 25 mM-ATP and 37.5 mM-2-mercaptoethanol and 1 μl of phage-T4 polynucleotide kinase (2 units; Pharmacia) solution, and the mixture was incubated for 10 min at 37 °C.

Calf intestine alkaline phosphatase. A 10 μl volume of 50 mM-Tris/HCl buffer, pH 8.5, containing 10 mM-$MgCl_2$ and 0.1 mM-$ZnCl_2$, also containing 20 pmol of

oligonucleotide and 90 munits of calf intestine alkaline phosphatase (Sigma type VIIS) was incubated for 10 min at 37 °C.

Electrophoresis on denaturing 20% polyacrylamide gel

The denaturing gel was prepared from 60 g of urea, 60 ml of 38% acrylamide, 2% of bisacrylamide solution, 12 ml of Tris/borate buffer, pH 8.3, containing 20 mM-EDTA, 4 ml of water, 800 μl of 10% (w/v) ammonium persulphate and 36 μl of tetramethylenediamine. To be able to analyse oligonucleotides with 5′-terminal AP sites, the samples must be either reduced with $NaBH_4$ or treated with methoxyamine, otherwise the loss of the 5′-terminal AP site is complete. The sample was exposed to 25 mM-methoxyamine for 10 min at 0 °C, and the solution was subsequently diluted with at least twice its volume of stop solution (90% formamide in 0.1 M-Tris/borate buffer, pH 8.3, containing 2 mM-EDTA, 0.02% Xylene Cyanol and 0.02% Bromophenol Blue). Samples (10 μl) were placed in the wells (12 mm diam.); the vertical electrophoresis was carried out at 900 V for either 2.5 h (the Bromophenol Blue had migrated 7 cm) or 14 h (the Bromophenol Blue had migrated 38 cm). The gels were autoradiographed with Fuji X-ray films with the use of a Kodax X-Omatic superfast intensifying screen.

Miscellaneous

Spermine and methoxyamine (both from Sigma Chemical Co.) were neutralized with HCl and with NaOH respectively; the solutions were kept at −20 °C.

Chromatography on DEAE-Sephadex A25 was as described by Bailly & Verly (1987). The phage-ϕX174 RF [^{3}H]DNA was analysed by electrophoresis on 1% agarose gel as described by Goffin & Verly (1982) in order to determine the average number of strand interruptions per molecule.

RESULTS

Nicking of the DNA strand near the AP sites by *E. coli* endonuclease IV is followed by another reaction

When [d(−),5′-^{32}P]DNA was submitted to a strong alkaline treatment that nicked near each AP site, 4% of the ^{32}P was released in acid-soluble [soluble in 5% (v/v) $HClO_4$ at 0 °C] molecules. The DNA fragmentation yielded oligonucleotides, some of them small enough to be acid-soluble. But the ^{32}P was in the 3′-phosphate ends of these oligonucleotides; indeed, all the ^{32}P was still adsorbable on Norit, and it could be released as inorganic phosphate by a 3′-phosphatase.

When [d(−),5′-^{32}P]DNA was incubated with *E. coli* endonuclease IV at 37 °C, the acid-soluble ^{32}P rapidly increased beyond 4% (Fig. 1), indicating that something occurred beyond the fragmentation at the AP sites; after 2 h the acid-soluble ^{32}P was about 50%, and most of it could not be adsorbed on Norit.

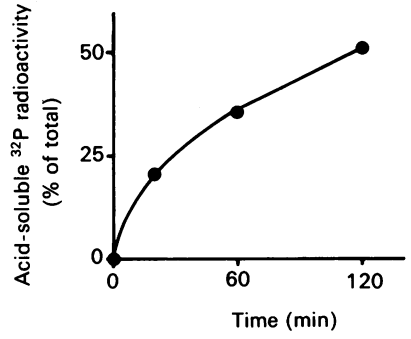

Fig. 1. Action of *E. coli* endonuclease IV on an AP-site containing DNA is not limited to the hydrolysis of the $C_{(3')}$-O–P bond 5′ to the AP sites

[d(−),5′-^{32}P]DNA was incubated with *E. coli* endonuclease IV and the acid-soluble ^{32}P (as percentage of total radioactivity) was determined after increasing times.

Nature of the 5′-end resulting from the nicking near AP sites by *E. coli* endonuclease IV

[^{32}P]pdT$_8$d(−)dT$_7$, hybridized to poly(dA), was submitted to digestion with *E. coli* endonuclease IV, and the 5′-labelled fragment was analysed by gel electrophoresis followed by autoradiography (Fig. 2). Several samples were treated in parallel to provide reference markers, and these are presented first.

Lanes 1 and 2 show [^{32}P]pdT$_8$dGdT$_7$ and [^{32}P]-pdT$_8$d(−)dT$_7$ respectively.

Lane 3 shows [^{32}P]pdT$_8$d(−)dT$_7$, hybridized to poly(dA), partially digested by *E. coli* endonuclease VI. The enzyme hydrolysed the $C_{(3')}$-O–P bond 5′ to the AP site (Gossard & Verly, 1978) to yield [^{32}P]pdT$_8$, which was subsequently partially degraded by the 3′→5′

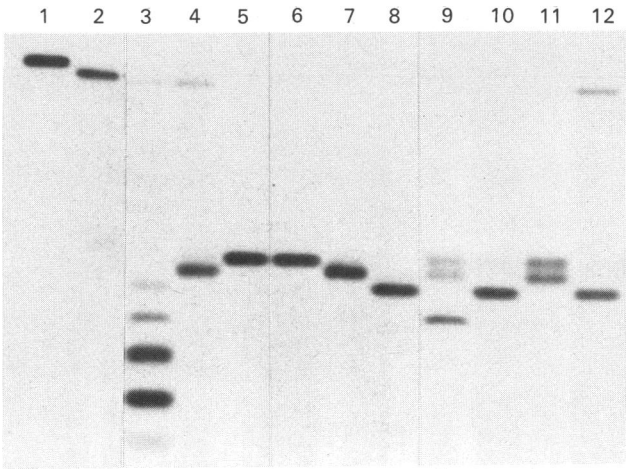

Fig. 2. Action of *E. coli* endonuclease IV on a 5′-labelled oligonucleotide containing an AP site

Lanes 1 and 2, [^{32}P]pdT$_8$dGdT$_7$ (lane 1) and [^{32}P]pdT$_8$-d(−)dT$_7$ (lane 2). Lane 3, [^{32}P]pdT$_8$d(−)dT$_7$, hybridized to poly(dA), partially digested by *E. coli* endonuclease VI/exonuclease III. Lane 4, [^{32}P]pdT$_8$d(−)dT$_7$ digested with snake-venom phosphodiesterase. Lanes 5 and 6, [^{32}P]pdT$_8$dA (lane 5), hybridized to poly(dA), submitted to digestion with *E. coli* endonuclease IV (lane 6). Lanes 7 and 8, [^{32}P]pdT$_8$d(−) (lane 7), hybridized to poly(dA), incubated with *E. coli* endonuclease IV (lane 8). Lanes 9 and 10, [^{32}P]pdT$_8$d(−)dT$_7$ submitted to a mild alkaline treatment (lane 9) and, after hybridization with poly(dA), to an incubation with *E. coli* endonuclease IV (lane 10). Lanes 11 and 12, [^{32}P]pdT$_8$d(−)dT$_7$, hybridized to poly(dA), incubated with *E. coli* endonuclease III (lane 11) or *E. coli* endonuclease IV (lane 12). Analysis by electrophoresis on polyacrylamide gel was followed by autoradiography.

exonuclease activity of the enzyme (exonuclease III), yielding four additional steps on the autoradiogram.

Lane 4 shows [^{32}P]pdT$_8$d(–)dT$_7$ incubated with snake-venom phosphodiesterase. The enzyme action stopped immediately before the AP site to give [^{32}P]pdT$_8$d(–) (see also Takeshita *et al.*, 1987). Some [^{32}P]pdT$_8$d(–)dT$_7$ was still intact, which underscores the processive activity of the enzyme.

Lane 12 shows [^{32}P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), incubated with *E. coli* endonuclease IV. The labelled substrate has been nicked to yield [^{32}P]pdT$_8$. This indicates that *E. coli* endonuclease IV nicks the $C_{(3')}$-O–P bond 5′ to the AP site.

E. coli endonuclease IV releases a 3′-terminal base-free deoxyribose 5′-phosphate or the 3′-terminal base-free 2′,3′-unsaturated sugar resulting from the nicking 3′ to an AP site by β-elimination. *E. coli* endonuclease IV is a 3′-phosphatase

The results are included in Fig. 2.

Lanes 5 and 7 show [^{32}P]pdT$_8$dA and [^{32}P]pdT$_8$d(–) respectively.

Lanes 6 and 8 show that *E. coli* endonuclease IV was without action on [^{32}P]pdT$_8$dA hybridized to poly(dA) (lane 6), but transformed [^{32}P]pdT$_8$d(–), also hybridized to poly(dA), into [^{32}P]pdT$_8$ (lane 8). Thus *E. coli* endonuclease IV can release a 3′-terminal base-free deoxyribose 5′-phosphate.

Lane 9 shows [^{32}P]pdT$_8$d(–)dT$_7$ submitted to a mild alkaline treatment. One can see the two products from the β-elimination reaction forming a doublet, which we represent by [^{32}P]pdT$_8$s(=), at the level of [^{32}P]pdT$_8$d(–) (lane 7), and the product of a subsequent partial δ-elimination, [^{32}P]pdT$_8$p (see also Bailly & Verly, 1987).

For lane 11 [^{32}P]pdT$_8$d(–)dT$_7$, hybridized to poly(dA), was incubated with *E. coli* endonuclease III, which is known to nick 3′ to AP sites by β-elimination (Bailly & Verly, 1987): one can see the doublet of β-elimination.

Lane 10 shows [^{32}P]pdT$_8$d(–)dT$_7$ submitted to a mild alkaline treatment (as for lane 9) and, after addition of poly(dA), incubated with *E. coli* endonuclease IV. There is a single band of [^{32}P]pdT$_8$. Comparison with lane 9 shows that the products of β-elimination are a substrate for *E. coli* endonuclease IV: [^{32}P]pdT$_8$s(=) was transformed into [^{32}P]pdT$_8$; in other words, the 3′-terminal base-free 2′,3′-unsaturated sugar 5′-phosphate was removed. Comparison with lane 9 also shows that [^{32}P]pdT$_8$p, the product of βδ-elimination, was transformed into [^{32}P]pdT$_8$. Another autoradiogram (not shown) presented a simpler situation: [^{32}P]pdT$_8$d(–)dT$_7$, submitted to a strong alkaline treatment to yield only [^{32}P]pdT$_8$p, was subsequently exposed to *E. coli* endonuclease IV; the [^{32}P]pdT$_8$p band was replaced by another band of [^{32}P]pdT$_8$. This clearly indicates that *E. coli* endonuclease IV hydrolyses a 3′-phosphate, but not a 5′-phosphate: the enzyme is a 3′-phosphatase.

Nature of the 3′-end of the interruption produced near an AP site by *E. coli* endonuclease IV

dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA, hybridized to poly(dA), was submitted to digestion by *E. coli* endonuclease IV; after addition of methoxyamine to stabilize the AP site, the 3′-labelled fragment was analysed by gel electrophoresis followed by autoradiography (Fig. 3). Several samples

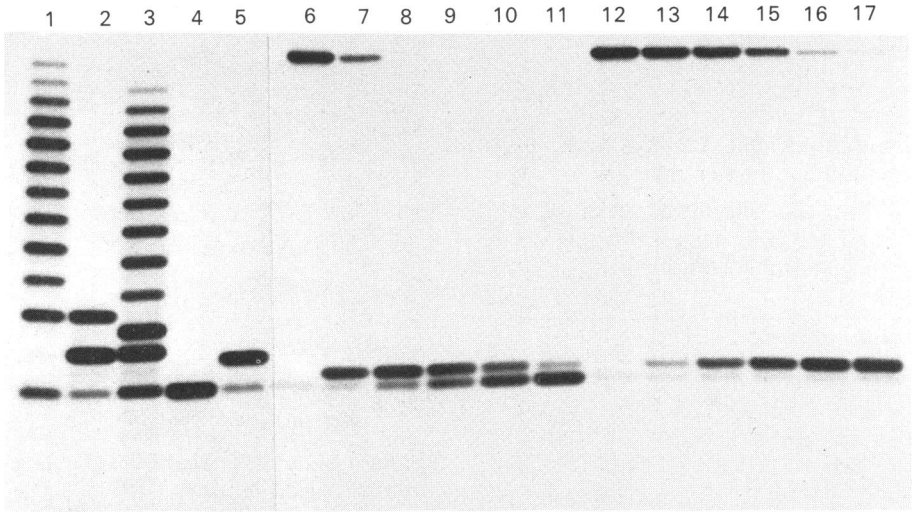

Fig. 3. Action of *E. coli* endonuclease IV on a 3′-labelled oligonucleotide containing an AP site

Lanes 1–3, a mixture of two samples of dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA digested with 5 munits and 10 munits of calf spleen phosphodiesterase in the presence of methoxyamine (lane 1); the same labelled oligonucleotide was digested with 40 munits of the enzyme also in the presence of methoxyamine (lane 2); the three digested samples were mixed and 5′-phosphorylated with ATP and phage-T4 polynucleotide kinase (lane 3). Lanes 4 and 5, dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA submitted to a strong alkaline treatment (lane 4), then incubated with calf intestine alkaline phosphatase (lane 5). Lane 6, dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA incubated without enzyme for 240 min at 37 °C. Lanes 7–11, dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA, hybridized to poly(dA), incubated at 37 °C with *E. coli* endonuclease IV for 10 min (lane 7), 30 min (lane 8), 60 min (lane 9), 120 min (lane 10) or 240 min (lane 11). Lane 12, dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA incubated without enzyme for 240 min at 37 °C with 25 mM-methoxyamine. Lanes 13–17, dT$_{11}$d(–)dT$_{11}$[^{32}P]ddA, hybridized to poly(dA), incubated at 37 °C with *E. coli* endonuclease IV, in the presence of 25 mM-methoxyamine, for 10 min (lane 13), 30 min (lane 14), 60 min (lane 15), 120 min (lane 16) or 240 min (lane 17). Analysis by electrophoresis on polyacrylamide gel was followed by autoradiography.

were treated in parallel to have reference markers, and these are presented first.

Lanes 1 and 2 show $dT_{11}d(-)dT_{11}[^{32}P]ddA$ partially degraded with calf spleen phosphodiesterase in the presence of methoxyamine. Lane 1 presents a mixture of two samples, one treated with 5 munits and the other with 10 munits of enzyme; besides a lower band of $pdT_{11}[^{32}P]$-ddA due to a partial degradation of the substrate by β-elimination, one has a ladder of 11 steps from $dT_{11}d(-)dT_{11}[^{32}P]ddA$ to $dTd(-)dT_{11}[^{32}P]ddA$. For lane 2 the sample was treated with 40 enzyme munits; besides the band of β-elimination, there are two steps corresponding to $dTd(-)dT_{11}[^{32}P]ddA$ and $d(-)dT_{11}[^{32}P]$-ddA.

For lane 3 the products in the mixture of the three samples of $dT_{11}d(-)dT_{11}[^{32}P]ddA$ treated with different amounts of calf spleen phosphodiesterase were 5′-phosphorylated with ATP and phage-T4 polynucleotide kinase; one sees a ladder of 13 rungs, one of them out of step; this is due to the fact that $d(-)dT_{11}[^{32}P]ddA$ is a poor substrate for the phage-T4 polynucleotide kinase. The other 12 rungs are the 11 ones going from $pdT_{11}d(-)dT_{11}[^{32}P]ddA$ to $pdTd(-)dT_{11}[^{32}P]ddA$, and $pdT_{11}[^{32}P]ddA$.

For lanes 4 and 5 $dT_{11}d(-)dT_{11}[^{32}P]ddA$ was submitted to a strong alkaline treatment to obtain $pdT_{11}[^{32}P]ddA$ (lane 4), then submitted to digestion with calf intestine alkaline phosphatase, which hydrolysed most of the $pdT_{11}[^{32}P]ddA$ into $dT_{11}[^{32}P]ddA$ (lane 5).

Lanes 6 and 12 show $dT_{11}d(-)dT_{11}[^{32}P]ddA$, hybridized to poly(dA), incubated for 240 min at 37 °C in the absence of enzyme, without (lane 6) or with 25 mM-methoxyamine (lane 12). The labelled substrate was quite stable in both conditions.

Lanes 7–11 show $dT_{11}d(-)dT_{11}[^{32}P]ddA$, hybridized to poly(dA), incubated at 37 °C with *E. coli* endonuclease IV for 10 min (lane 7), 30 min (lane 8), 60 min (lane 9), 120 min (lane 10) or 240 min (lane 11). The labelled substrate had disappeared after 30 min; it began to be cut into $pd(-)dT_{11}[^{32}P]ddA$, but this first product was progressively replaced by $pdT_{11}[^{32}P]ddA$. Thus, after a rapid cleavage of the $C_{(3')}$-O–P bond 5′ to the AP site, there was a slower rupture of the $C_{(3')}$-O–P bond 3′ to the AP site.

For lanes 13–17 the experiment was carried out as for lanes 7–11 but in the presence of 25 mM-methoxyamine. The addition of methoxyamine to the AP site (Talpaert-Borlé & Liuzzi, 1983) did not prevent the cleavage of the $C_{(3')}$-O–P bond 5′ to the AP site by *E. coli* endonuclease IV, although the reaction rate was decreased. On the other hand, it suppressed the rupture of the $C_{(3')}$O–P bond 3′ to the AP site.

Base-free sugar phosphate released by *E. coli* endonuclease IV acting on an AP site in DNA

[d(–),1′,2′-^{3}H,5′-^{32}P]DNA (100 μg) and 1 unit of *E. coli* endonuclease IV in 1 ml of buffer were incubated for 120 min at 37 °C. After addition of 10 μmol of deoxyribose 5-phosphate and 1 μmol of dUMP, the mixture was chromatographed on DEAE-Sephadex. Fig. 4 shows that the ^{32}P radioactivity emerged as a single peak delayed relative to the deoxyribose 5-phosphate marker. On the other hand, the ^{3}H radioactivity formed two peaks: one contained tritiated molecules not retained by the column and that were volatile, the other was coincident with the ^{32}P peak.

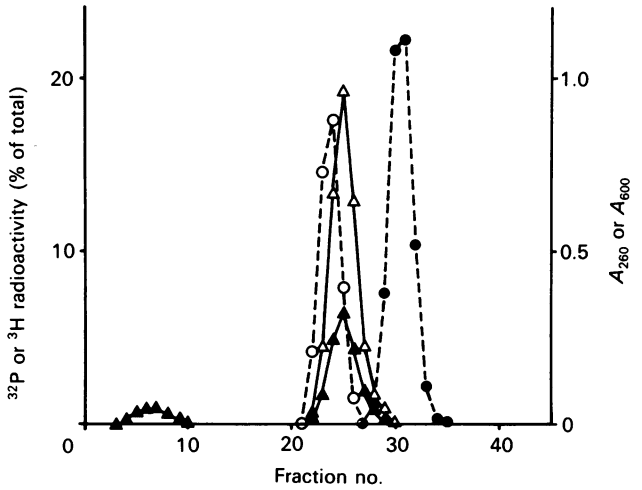

Fig. 4. DEAE-Sephadex analysis of the sugar phosphate released from AP-site-containing DNA by *E. coli* endonuclease IV

[d(–),1′,2′-^{3}H,5′-^{32}P]DNA was incubated with *E. coli* endonuclease IV. After addition of deoxyribose 5-phosphate and dUMP as elution markers, the solution was chromatographed on DEAE-Sephadex. The ^{32}P (△) and ^{3}H (▲) radioactivities were measured on the collected fractions; they are expressed as percentages of the respective total radioactivities. The dUMP (●) was measured at A_{260}, whereas the deoxyribose 5-phosphate (○) was measured at A_{600} after reaction with diphenylamine.

The cleavage of the $C_{(3')}$-O–P bond 5′ to the AP site is probably the result of a hydrolysis. But the cleavage of the $C_{(3')}$-O–P bond 3′ to the AP site cannot be a hydrolysis, which would have released a doubly labelled deoxyribose 5-phosphate with a $^3H/^{32}P$ ratio identical with that of the substrate DNA. We have shown that the sugar phosphate released by *E. coli* endonuclease IV has a chromatographic behaviour that was different from that of deoxyribose 5-phosphate; moreover, the $^3H/^{32}P$ ratio was lower than that of the substrate DNA. The lost ^{3}H was found in volatile molecules, probably tritiated water. The cleavage of the $C_{(3')}$-O–P bond 3′ to the AP site is thus probably the result of a β-elimination, which released as H^+ a labelled hydrogen from the 2′-position of the base-free deoxyribose.

Nicking of the $C_{(3')}$-O–P bond 3′ to the AP site that occurs after the hydrolysis of the $C_{(3')}$-O–P bond 5′ to the AP site is a spontaneous reaction in which *E. coli* endonuclease IV plays no role: the reaction can, however, be accelerated with spermine

$[^{32}P]pd(-)dT_{11}$, hybridized to poly(dA), was incubated at 37 °C for 30, 120 or 240 min, with or without *E. coli* endonuclease IV. Fig. 5 presents the results either after a long (Fig. 5*b*) or a short (Fig. 5*a*) electrophoresis preceded by a methoxyamine treatment. After the long electrophoresis, one sees a progressive disappearance of the labelled substrate, but the rate of disappearance is the same whether the enzyme is present (lanes 7–9) or not (lanes 4–6). After the short electrophoresis, one sees the progressive appearance of the labelled sugar phosphates; the results are similar with or without *E. coli* endonuclease IV. The released sugar phosphates formed two bands.

$[^{32}P]pd(-)dT_{11}$, hybridized to poly(dA), was incubated for 10 min in a pH 8.0 buffer without or with 0.2 mM-

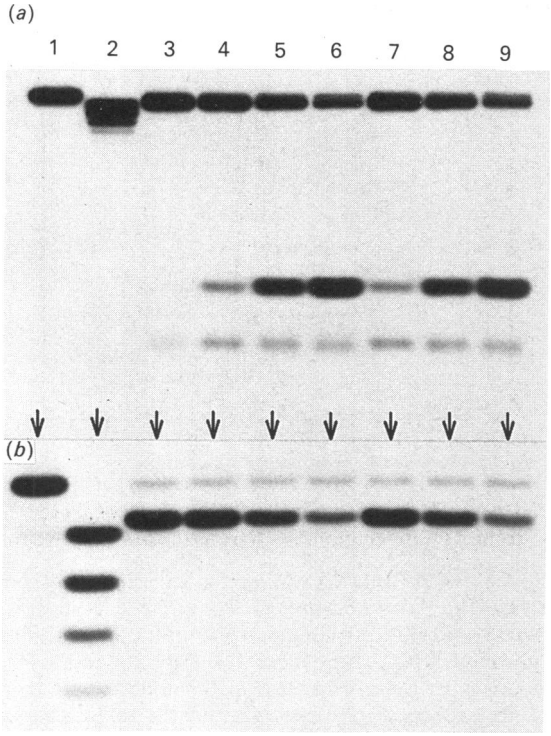

Fig. 5. After *E. coli* endonuclease IV has hydrolysed the $C_{(3')}$-O–P bond 5′ to an AP site in a polydeoxynucleotide, the rupture of the $C_{(3')}$-O–P bond 3′ to the AP site is a spontaneous reaction

Lanes 1–3, [^{32}P]pdGdT_{11} (lane 1), [^{32}P]pdT_{8-11} (lane 2) and [^{32}P]pd(–)dT_{11} (lane 3). Lanes 4–9, [^{32}P]pd(–)dT_{11}, hybridized to poly(dA), incubated for 30 min (lane 4), 120 min (lane 5) or 240 min (lane 6) without enzyme, or for 30 min (lane 7), 120 min (lane 8) or 240 min (lane 9) with *E. coli* endonuclease IV. Analysis by electrophoresis on polyacrylamide gel was followed by autoradiography. The samples were loaded twice in the same wells. After a first loading, the electrophoresis was carried out for 11.5 h and, after the second loading, for an additional 2.5 h. Thus (*a*) corresponds to the short electrophoresis (2.5 h) and (*b*) to the long electrophoresis (14 h).

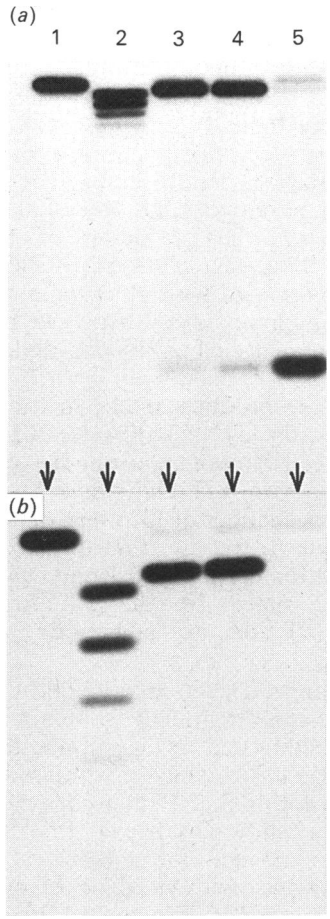

Fig. 6. Lability of 5′-terminal base-free deoxyribose 5-phosphate is considerably increased by spermine

Lanes 1–3, [^{32}P]pdGdT_{11} (lane 1), [^{32}P]pdT_{8-11} (lane 2) and [^{32}P]pd(–)dT_{11} (lane 3). Lanes 4 and 5, [^{32}P]pd(–)dT_{11} was incubated for 10 min at 37 °C and pH 8.0, without spermine (lane 4) or with 0.2 mM-spermine (lane 5). The electrophoresis was carried out as described for Fig. 5.

spermine. Fig. 6 shows that the disappearance of the substrate (Fig. 6*b*) and the appearance of the sugar phosphate (Fig. 6*a*) were considerably accelerated by the presence of spermine (lane 5 to be compared with lane 4).

After hydrolysis of the $C_{(3')}$-O–P bond 5′ to the AP site with an AP endonuclease, no 5′→3′ exonuclease is absolutely required to repair the AP site

A mixture of 9 μl of 50 mM-Hepps/KOH buffer, pH 8.0, containing 1 mM-EDTA and 0.2 mM-NaCl, also containing 2.5 μg of partially depurinated phage-φX174 RF-I [^{3}H]DNA (with an average of 1.30 intact AP sites and 0.08 parasitic break per molecule), and 2 μl of *E. coli* endonuclease IV or *E. coli* endonuclease III preparation was incubated at 37 °C. After deproteinization with phenol, followed by extraction with diethyl ether, 35 μl of a reaction medium was added so that the final concentrations were 50 mM-Hepps/KOH buffer, pH 8.0, 10 mM-$MgCl_2$, 1 mM-dithiothreitol, 40 μM-dATP, -dGTP, -dCTP and -dTTP, and 400 μM-ATP. The medium was completed with 5 μl of 50 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol and 50 % (v/v) glycerol, also containing 2.5 units of the Klenow fragment of DNA polymerase I (Boehringer), and 1 μl of a solution containing 10 units of phage-T4 DNA ligase (Pharmacia), and incubated for 2 h at 12 °C. Controls were carried out without one or several enzymes. Each sample was divided in two; one part was treated with an excess of rat liver AP endonuclease. The two parts were deproteinized and analysed by electrophoresis on agarose gels in order to determine the average number of strand interruptions per RF DNA molecule (for details see Bailly & Verly, 1988).

Table 1 shows that *E. coli* endonuclease IV introduced a strand interruption near each AP site (column 1) and that a subsequent treatment with the Klenow polymerase and phage-T4 DNA ligase (column 2) led to the disappearance of a part of these interruptions (1.34→0.58); no intact AP site re-appeared during this repair, as shown by a subsequent treatment with an AP endonuclease.

We have carried out a similar experiment with the same results using the chromatin AP endonuclease from rat liver instead of *E. coli* endonuclease IV and DNA

Table 1. Repair of partially depurinated bacteriophage-ϕX174 RF-1 DNA by the use of *E. coli* endonuclease IV, Klenow polymerase and bacteriophage-T4 DNA ligase

Partially depurinated phage-ϕX174 RF-I [^{3}H]DNA was treated with *E. coli* endonuclease IV or *E. coli* endonuclease III, then with the Klenow fragment of DNA polymerase I and phage-T4 DNA ligase. Controls were carried out without enzymes. The average number n of strand interruptions per RF molecule was calculated from the results of agarose-gel electrophoresis. A portion of each sample was submitted to an additional treatment with rat liver AP endonuclease before it was electrophoresed to determine the average number n' of strand interruptions.

Column...	0	1	2	3
Endonuclease IV	−	+	+	−
Endonuclease III	−	−	−	+
DNA polymerase	−	−	+	+
DNA ligase	−	−	+	+
n	0.13	1.34	0.58	1.25
AP endonuclease	+	+	+	+
n'	1.40	1.36	0.62	1.30

polymerase β, also from rat liver chromatin, instead of the Klenow polymerase (results not shown).

For the sake of comparison, we show (column 3) that *E. coli* endonuclease III, which nicks only 3′ to the AP site by β-elimination, cannot replace *E. coli* endonuclease IV in the repair experiment.

DISCUSSION

The literature presents *E. coli* endonuclease IV as an enzyme that hydrolyses the phosphodiester bond 5′ to AP sites in DNA, leaving 3′-OH and 5′-phosphate ends. The main argument is that the 3′-OH end can efficiently prime DNA polymerases (Mosbaugh & Linn, 1982). Another indirect result was given by Takeshita *et al.* (1987), who showed that *E. coli* endonuclease IV nicked a synthetic 5′-labelled oligonucleotide on the 5′ side of an abasic site where the deoxyribose residue had been replaced by a tetrahydrofuran residue. Using oligonucleotides containing an AP site, hybridized to a complementary strand, we indeed show that the $C_{(3')}$-O–P bond 5′ to AP site is nicked to give the expected 5′-fragment and that the primary 3′ fragment had a base-free sugar 5-phosphate at its 5′-end. We have also shown that *E. coli* endonuclease IV can release a 3′-terminal base-free deoxyribose 5′-phosphate or a 3′-terminal base-free unsaturated sugar 5-phosphate produced by nicking 3′ to the AP site by β-elimination. This latter observation explains why the unproductive 3′-end produced by the so-called AP endonucleases class I, which are β-elimination catalysts (Bailly & Verly, 1984), can be activated by *E. coli* endonuclease IV to become a primer for *E. coli* DNA polymerase I (Mosbaugh & Linn, 1982).

Starting with the idea that the only action of *E. coli* endonuclease IV on an AP site was to hydrolyse the $C_{(3')}$-O–P bond 5′ to the lesion, we were astonished that, when a DNA containing AP sites labelled with ^{32}P on the 5′ side was incubated with the enzyme, the acid-soluble ^{32}P radioactivity became rapidly greater than the 4% expected from a simple fragmentation of the DNA strands at the AP sites. Indeed, the acid-soluble ^{32}P radioactivity increased to more than 50% in 120 min, and this result could not be explained by the release of inorganic [^{32}P]phosphate; indeed, the *E. coli* endonuclease IV preparation was free of 5′-phosphatase activity. We obtained the explanation when we observed the action of *E. coli* endonuclease IV on an oligonucleotide containing an AP site and labelled at its 3′-end: the 3′ fragment with a base-free deoxyribose 5′-phosphate at its 5′-end was only a primary product; it rapidly lost the base-free sugar phosphate; the hydrolysis of the $C_{(3')}$-O–P bond 5′ to the AP site was followed by the rupture of the $C_{(3')}$-O–P bond 3′ to the AP site. The second step escaped observation as long as randomly labelled DNA containing AP sites was used as a substrate for the enzyme.

In order to understand the mechanism of this second cleavage, DNA containing AP sites labelled with ^{3}H in the 1′- and 2′-positions of the base-free deoxyribose and with ^{32}P 5′ to this residue was incubated with *E. coli* endonuclease IV and the reaction products were analysed on DEAE-Sephadex. A doubly labelled sugar phosphate emerged from the column, but in a position different from that of a deoxyribose 5-phosphate marker; moreover, the sugar had lost some ^{3}H, which was found in molecules not retained by the column and which were volatile. All these results indicate that the $C_{(3')}$-O–P bond 3′ to the AP site was not hydrolysed, but they agree with the hypothesis that the 5′-terminal base-free sugar 5-phosphate was lost by β-elimination; such a mechanism would release as H^+ a ^{3}H-labelled hydrogen (to become volatile ^3H_2O) from the 2′-position and a 2′,3′-unsaturated sugar 5-phosphate containing all the ^{32}P but only the residual ^{3}H.

We then observed that *E. coli* endonuclease IV had nothing to do with the cleavage of the $C_{(3')}$-O–P bond 3′ to the AP site; it is a spontaneous reaction. Whereas the half-life of an internal AP site has been estimated to be 200 h at 37 °C (Lindahl & Anderson, 1972), the half-life of a 5′-terminal base-free deoxyribose 5-phosphate is only about 2 h (see Fig. 1). This very short half-life led us to postulate that, after nicking 5′ to an AP site with an AP endonuclease, no 5′→3′ exonuclease is needed to remove the lesion; indeed, the AP site falls off spontaneously. Using phage-ϕX174 RF-1 DNA containing an AP site, we have shown that the lesion can be removed and the RF molecule completely repaired with only three enzymes: an AP endonuclease (*E. coli* endonuclease IV or the chromatin AP endonuclease from rat liver), a DNA polymerase devoid of 5′→3′ exonuclease activity (Klenow polymerase or rat liver DNA polymerase β) and DNA ligase.

The success of this repair does not mean that the loss of the 5′-terminal AP site cannot be accelerated *in vivo* by β-elimination catalysts or by an enzymic process. Using DNA with AP sites labelled by reduction with tritiated $NaBH_4$, Gossard & Verly (1978) observed that, after a 5′ incision with *E. coli* endonuclease VI, the AP sites were excised by the 5′→3′ exonuclease activity of DNA polymerase I. Of course, reduction prevented the nicking by β-elimination, but the rate of loss of the reduced AP site was much higher than what is observed with the non-reduced lesion in absence of an exonuclease. The situation is different in mammalian cells, where no enzyme has been demonstrated to hydrolyse a phosphodiester bond

3′ to an AP site; in chromatin, histones and polyamines might catalyse the β-elimination (Bailly & Verly, 1988). We indeed show in the present paper that spermine highly accelerates the loss of the 5′-terminal base-free deoxyribose 5′-phosphate.

E. coli endonuclease IV has other activities that enable it to participate in the repair of strand breaks produced by ionizing radiations; such breaks are limited by 5′-phosphate and 3′-phosphate or 3′-phosphoglycollate ends (Henner *et al.*, 1982), and the first step of the repair of these breaks is believed to be the hydrolysis of the abnormal 3′-ends into 3′-OH groups that can be used by a DNA polymerase. Demple *et al.* (1986) and Siwek *et al.* (1988) have shown that *E. coli* endonuclease IV is a 3′-phosphoglycollatase; we show in the present paper that it is also a 3′-phosphatase, and Levin *et al.* (1988) have also come to the same conclusion. The 3′-phosphatase activity of *E. coli* endonuclease IV may also play a role in the repair of gaps limited by 3′-phosphate and 5′-phosphate produced by the excision of AP sites by a $\beta\delta$-elimination reaction.

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