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Excision of Apurinic Sites from DNA with Enzymes Isolated from Rat-Liver Chromatin

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Apurinic sites were excised from ϕX174 RF DNA with two enzymes isolated from rat liver chromatin: an apurinic/apyrimidinic endodeoxyribonuclease and a 5′-3′-exonuclease; the resulting gap was filled with DNA polymerase β also prepared from rat liver chromatin and the repair was fully terminated with T4 ligase.

In 1974, Verly et al. [1] repaired *in vitro* T7 phage DNA containing apurinic sites with enzymes isolated from *Escherichia coli*: endonuclease VI, which is the major AP endodeoxyribonuclease of the bacterium, DNA polymerase I and ligase (from T4-infected *E. coli*). Gossard and Verly [2,3] have worked out the details of the repair: endonuclease VI hydrolyzes a phosphoester bond on the 5′ side of the apurinic site leaving a 3′-OH and a 5′-phosphate; the exonuclease III activity of endonuclease VI degrades the nicked strand in the 3′-5′ direction forming a gap; DNA polymerase I starts from the 3′-OH end left by exonuclease III, fills the gap, excises the apurinic site in a dinucleotide or trinucleotide, catalyzes the translation of a nick in the 5′-3′ direction; this nick is finally sealed by ligase.

In 1978, Bose et al. [4] repaired T7 phage alkylated DNA containing apurinic sites with human lymphoblast enzymes and T4 ligase. They used DNA polymerase α and an AP endodeoxyribonuclease with an associated 5′-3′ exonuclease activity prepared from a total cellular extract.

Recent work from our laboratory has shown that DNA repair enzymes are located in chromatin. We decided to use enzymes prepared from rat liver chromatin to repair DNA containing apurinic sites: the major chromatin AP endodeoxyribonuclease uncontaminated with any exonuclease activity, a 5′-3′ exonuclease and DNA polymerase β. For the ligation step, although rat liver chromatin contains a ligase, the amount isolated was too low to enable the demonstration of apurinic site excision; we were obliged to use T4 ligase.

MATERIALS AND METHODS

The Enzymes

Pancreatic deoxyribonuclease I and T4 ligase were obtained from Boehringer and B.R.L. respectively.

For the purification of the chromatin enzymes of rat liver, the nuclei were first isolated and the chromatin was prepared according to Thibodeau and Verly [5]. The chromatin was dissociated with heparin-Ultrogel and the enzymes extracted with 0.5 M KCl solution [6].

Abbreviations. AP, apurinic or apyrimidinic; RF, replicative form; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

Enzymes. Apurinic/apyrimidinic (AP) endodeoxyribonuclease (EC 3.1.25.2); pancreatic deoxyribonuclease I (EC 3.1.21.1); T4 DNA ligase (EC 6.5.1.1); DNA polymerase β (EC 2.7.7.7).

The chromatin AP endodeoxyribonuclease was supplied by R. César of our laboratory. The enzyme had been chromatographed successively on hydroxyapatite, phosphocellulose, heparin-Sepharose and DNA-cellulose. This enzyme is not the same as the one isolated by Thibodeau et al. [7] which might be a degradation product of the true chromatin enzyme. The purified endonuclease is strictly specific for AP sites and devoid of exonuclease activity; it hydrolyzes the phosphodiester bridge which is the immediate neighbour of the AP site on its 5′ side leaving a 3′-OH and a 5′-phosphate (César and Colson, unpublished). The enzyme preparation in buffer A (20 mM Tris/HCl, 1 mM EDTA, 0.2 mM dithiothreitol, pH 8.0), 10% glycerol, contained 53600 units/ml; one unit incises DNA near 1 pmol of AP sites/min at 37 °C [8].

The 5′-3′ exonuclease was isolated from chromatin using the method of Lindahl et al. [9] for DNase IV. This exonuclease, like DNase IV, is active on double-stranded DNA and degrades it from nicks in the 5′-3′ direction (Zocchi, unpublished). The enzyme preparation in buffer B, 50% glycerol, contained 32 units/ml; one unit, as defined by Lindahl et al. [9], releases 1 nmol of acid-soluble nucleotides from poly-[d(A-T)] in 15 min at 37 °C.

The DNA polymerase β was prepared from rat liver chromatin by R. Schyns of our laboratory. The enzyme was purified by two chromatographies on DNA-cellulose, one with double-stranded and the other with single-stranded DNA; the preparation was devoid of endonuclease and exonuclease activities. The enzyme preparation in buffer A, 40% glycerol, contained 38 units/ml; one unit incorporates 1 nmol thymidylate in DNA in 60 min in the conditions described by Chang [10].

The Substrates

Normal DNA. Tritium-labelled RF-I DNA of phage ϕX174, with a specific activity of 650000 dis · min^{-1} · μg^{-1}, was prepared and kept at 4 °C in 0.15 M NaCl, 0.015 M sodium citrate, 0.1 mM EDTA, pH 7.0, 0.1% benzyl alcohol, at a concentration of 120 µg/ml.

Nicked DNA. 400 µl of 50 mM Tris/HCl, 10 mM $MgCl_2$, pH 7.5, containing 24 µg of RF-I [^{3}H]DNA were incubated with 0.001 unit of deoxyribonuclease I for 12 min at 30 °C. The reaction was stopped with 100 µl 0.2 M EDTA, the proteins were extracted with phenol and the solution was dialyzed against buffer B (5 mM NaCl, 1.5 mM sodium acetate, 1 mM

EDTA, pH 7.0). This treatment produces an average of between 1 and 2 nicks per molecule of ϕX174 RF DNA.

Depurinated DNA. To 1 vol. of RF-I [^{3}H]DNA solution (100 µg/ml) was added 1 vol. of 0.2 M sodium acetate, pH 3.8. After a 60-min incubation at 37 °C, the pH was raised with 1 vol. of 1 M potassium phosphate, pH 7.0, and the solution dialyzed against buffer B. This treatment yields about 1 apurinic site per molecule of ϕX174 RF-I DNA.

Determination of the Number of Nicks, Gaps and Apurinic Sites in ϕX174 RF DNA

Two complementary methods were used.

Nitrocellulose Membrane Filtration. A few changes and precisions were brought to the method described by Radman [11]. To 10–20 µl of RF DNA solution (2 µg/ml) was added 1 ml of 0.9 M NaCl, 0.1 M K phosphate, 25 mM EDTA, pH 11.7; after a 60-min incubation at 30 °C, the pH was brought down to 8.0 with 0.5 ml of 2 M Tris/HCl, pH 4. This treatment hydrolyzes a phosphoester bond near each apurinic sites, but we checked that it does not introduce nicks in intact DNA, nor prevent the renaturation of RF-I DNA. On the other hand, it denatures irreversibly any RF DNA containing a nick, gap or apurinic site. 3 ml of 1 M NaCl, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0, were then added and the solution left 30 min at room temperature to reanneal the RF-I and RF-IV molecules, before placing the tubes in an ice bath. The cooled solution was poured onto a nitrocellulose filter (Schleicher & Schüll BA85) and the filtration rate adjusted at 5 ml/min; the filter was rinsed twice with 2 ml 0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, then with 5 ml 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0. The denatured DNA from RF-II and RF-III remains on the filter whereas the renatured DNA from RF-I or RF-IV passes into the filtrate. The filters were placed in counting vials; the DNA was digested in 0.5 ml of 1 M HCl at 50 °C for 1 h; after cooling to room temperature, 1 ml of ethyl acetate was added to dissolve the filter and, 15 min later, 10 ml of Lumagel for radioactivity determination. The average number n of nicks, gaps and apurinic sites per RF molecule is calculated from the fraction F of the total radioactivity retained on the filter using Poisson's equation:

$$1 - F = \frac{\text{radioactivity (RF-I + RF-IV)}}{\text{total radioactivity}} = e^{-n}.$$

Gel Electrophoresis. The DNA solution containing 0.1 % sodium dodecylsulfate was heated 3 min at 65 °C to prevent an action of the proteins on DNA migration; a solution of Ficoll, stained with bromophenol blue, was added to obtain a 2 % concentration of the polymer. Aliquots (35 µl) were placed in the wells of 3-mm-thick agarose gels containing ethidium bromide (2 % agarose; 1.5 µg ethidium bromide/ml) on plexiglass plates (12 × 13 cm). A 120-V tension was applied for 10 min; it was then lowered to 20 V (about 8 mA/plate) for 15 h. The electrophoresis separates the four ϕX174 RFs. To measure the radioactivity in each band, two procedures were followed: fluorography or collection for radioactivity determination in a counter. For fluorography, about 10 ng of DNA (around 6500 dis · min^{-1}) were placed in each well. After the electrophoresis, the gel was processed and the fluorography made as described by Laskey [12]. For the other method, each well received 500 ng of DNA. After electrophoresis, the gel was examined under ultraviolet light and the fluorescent bands were cut away and placed in counting vials. After addition of 0.5 ml of 0.5 M HCl, heating at 96 °C for 30 min and cooling, 10 ml of Lumagel was added for radioactivity determination. Since there is no alkaline treatment, the technique, as described so far, does not detect apurinic sites. The average number of nicks and gaps per RF molecule is given by the same equation as above. RF-IV can however be distinguished from RF-I: RF-IV may arise from relaxation of RF-I by a topoisomerase or from repair of RF-II molecules. Sometimes, before the electrophoresis, the DNA was denatured in an alkaline cycle as described for the nitrocellulose filter assay. The denatured DNAs from RF-II and RF-III migrate in two bands faster than the renatured RF-I and RF-IV molecules. In this case, as in the nitrocellulose assay, n from Poisson's equation is the average sum of nicks, gaps and apurinic sites per RF DNA molecule.

RESULTS

Absence of Parasitic Endonucleases in the Enzyme Preparations

ϕX174 RF-I DNA was incubated with each enzyme preparation in the conditions used in the repair experiments. The number of nicks introduced by this treatment was determined either by filtration on nitrocellulose or by gel electrophoresis. For each enzyme preparation (AP endodeoxyribonuclease, 5′-3′ exonuclease, DNA polymerase β, and T4 ligase), this number per molecule was low enough. The conclusion was that these enzyme preparations could be used in an attempt to repair ϕX174 RF-I DNA containing apurinic sites. The results are not given since controls in the repair experiments give the same information.

No relaxing of RF-I DNA by T4 ligase was observed, but the DNA polymerase β preparation contained some topoisomerase activity. Gel electrophoresis in the presence of ethidium bromide showed a displacement of the RF-I band towards the RF-IV position. On the other hand, when ethidium bromide was not in the gel during the electrophoresis but added just before examination under ultraviolet light, a series of discrete bands were observed showing a partial relaxation of RF-I molecules. Because of this topoisomerase activity, appearance of a RF-IV band means DNA repair only when there are no RF-I molecules before addition of DNA polymerase β and ligase and, obviously, when the incubation mixture does not contain the DNA polymerase β preparation.

Testing the Enzymes with Nicked ϕx174 DNA

In several tubes, a solution of nicked ϕX174 RF DNA (15 µg/ml) and 5′-3′ exonuclease (3 units/ml) was incubated 20 min at 37 °C in buffer C (50 mM Epps, 15 mM $MgCl_2$, 1 mM dithiothreitol, 1 % bovine serum albumin, pH 8.0); the enzyme was then inactivated by a 5-min heating at 65 °C. Controls were carried out without enzyme. To each tube were added the four deoxynucleoside triphosphates and ATP to reach 40 µM and 0.4 mM final concentrations respectively, then, depending on the tubes, DNA polymerase β (6 units/ml) and T4 ligase (20 units/ml) or an equivalent volume of buffer. The DNA final concentration was 5 µg/ml. The mixtures were incubated either 75 min at 20 °C or 120 min at 12 °C (a low temperature was used to avoid a possible strand displacement during the polymerisation step). The reaction was

Table 1. *Repair of nicked φX174 RF DNA*
The indicated temperature is that used for the incubation with DNA polymerase β and ligase (120 min at 12 °C, or 75 min at 20 °C). *n* is the average number of nicks and gaps per φX174 RF DNA molecule

Exonuclease 5′-3′	DNA polymerase β	T4 ligase	Temperature	*n*
			°C	
−	−	−	12	1.43
+	−	−	12	1.75
−	+	−	12	1.66
−	−	+	12	0.40
+	−	+	12	1.08
+	+	+	12	0.50
+	−	+	20	1.04
+	+	+	20	0.52

Table 2. *Excision of apurinic sites from φX174 RF-I DNA*
Determined with the nitrocellulose filter assay, *n* is the average number of nicks, gaps and apurinic sites per φX174 RF DNA molecule. Gel electrophoreses performed without or with prior alkaline denaturation indicated that the RF DNAs used as substrates contained an average of 0.42 nick and 0.90 intact apurinic site per molecule in experiment (1), and 0.47 nick and 1.33 intact apurinic sites in experiment (2)

AP endonuclease	Exonuclease 5′-3′	DNA polymerase β	Ligase	*n* in expt 1	*n* in expt 2
−	−	−	−	1.22	1.67
+	−	−	−	1.44	1.97
−	−	−	+	1.09	1.38
+	−	−	+	1.17	1.64
+	+	−	+	1.60	1.91
+	+	+	+	0.55	0.78
−	+	−	+	1.64	1.92
−	+	+	+	0.58	0.89

stopped by adding EDTA to a 20 mM final concentration. To prevent any disturbance by proteins during the analytical step, sodium dodecylsulfate was added to 0.1% final concentration and the solution was heated at 65 °C for 5 min. The assay was carried out using nitrocellulose filters as described in Materials and Methods. The total radioactivity poured on each filter was 9700 dis · min^{-1}. The results given in Table 1 are the means of three determinations; the error on *n*, the average number of nicks or gaps, is less than ± 0.1.

The control, without enzymes, shows that the average number of nicks per φX174 RF molecule used as a substrate was 1.43. A treatment with the 5′-3′-exonuclease preparation or the DNA polymerase β preparation alone slightly increased the number of breaks (nicks and gaps). On the other hand, a treatment with T4 ligase alone resealed most of the nicks. However, if an exposure to the 5′-3′ exonuclease preceded the ligase treatment, the repair was much lower since the nicks transformed into gaps by the exonuclease could not be resealed by the ligase. When the three enzymes were used, the result was about the same as when the nicked DNA was directly incubated with ligase alone. This means that the gaps produced by the exonuclease could be repaired by the successive actions of DNA polymerase β and ligase. The results were the same whether the incubation with DNA polymerase β and ligase was carried out at 20 °C or 12 °C.

Excision of Apurinic Sites from φX174 RF-I DNA

Two independent experiments are presented in Table 2; they gave the same qualitative results. Only the first experiment will be discussed.

Analysis of the depurinated φX174 RF-I DNA by gel electrophoresis, without and with a previous alkaline cycle of denaturation, showed that this substrate contained an average of 0.42 nick and 0.90 intact apurinic site per molecule. A solution (100 µl) of 40 µg of this depurinated φX174 RF-I DNA and 250 units of chromatin AP endodeoxyribonuclease in buffer C was incubated for 15 min at 37 °C. The reaction was stopped by heating 5 min at 65 °C. A control was carried out without enzyme. Analysis by gel electrophoresis showed that the enzyme treatment introduced a nick next to most apurinic sites.

The treatments of the depurinated φX174 RF-I DNA, nicked or not by the AP endodeoxyribonuclease, by the chromatin 5′-3′ exonuclease, DNA polymerase β and T4 ligase, were performed as described for the repair of nicked DNA in the previous section. The incubation with the chromatin DNA polymerase β and T4 ligase was at 12 °C for 120 min. The results are in Table 2. Because of the alkaline cycle of denaturation in the nitrocellulose filter assay, *n* is the average number of nicks, gaps and intact apurinic sites per φX174 RF DNA molecule.

The control without enzymes shows that the average number of nicks and apurinic sites per molecule of substrate was 1.22; this is in good agreement with the value obtained by gel electrophoresis (1.32, see above). A treatment with the chromatin AP endodeoxyribonuclease slightly increased this value. Incubation with T4 ligase alone decreased the total number of nicks and apurinic sites; this decrease (1.22 − 1.09 = 0.13) was lower than the number of nicks in the substrate (0.42) so that it can be concluded that ligase repaired some of those parasitic breaks but not all of them. When there was an incubation with the AP endodeoxyribonuclease before the treatment with ligase, the decrease in total number of nicks and apurinic sites was nearly the same as when there was no exposure to the AP endodeoxyribonuclease; this was expected since the assay does not differentiate between intact apurinic sites and apurinic sites associated with single-strand breaks.

However, there is an interesting question that can be solved using gel electrophoresis: might the nick, produced by the AP endodeoxyribonuclease on the 5′ side of the apurinic site, be resealed by T4 ligase since it is limited by a 3′-OH and a 5′-phosphate? Fig. 1A shows that it seems to be the case. The DNAs have not been submitted to the alkaline cycle of denaturation so that the apurinic sites which were not already associated with nicks remained intact. Lane a indicates that the depurinated φX174 RF DNA contained some parasitic breaks; lane b shows that most of these breaks, probably caused by radiolysis, were not repaired by ligase; an incubation with the AP endodeoxyribonuclease producing nicks near apurinic sites converted many RF-I molecules of the substrate into RF-II (lane c); subsequent treatment with T4 ligase repaired RF-II molecules into RF-IV (lane d). Since the AP endodeoxyribonuclease preparation had little action on intact strands, it must be concluded that nicks produced near apurinic sites were repaired by T4 ligase.

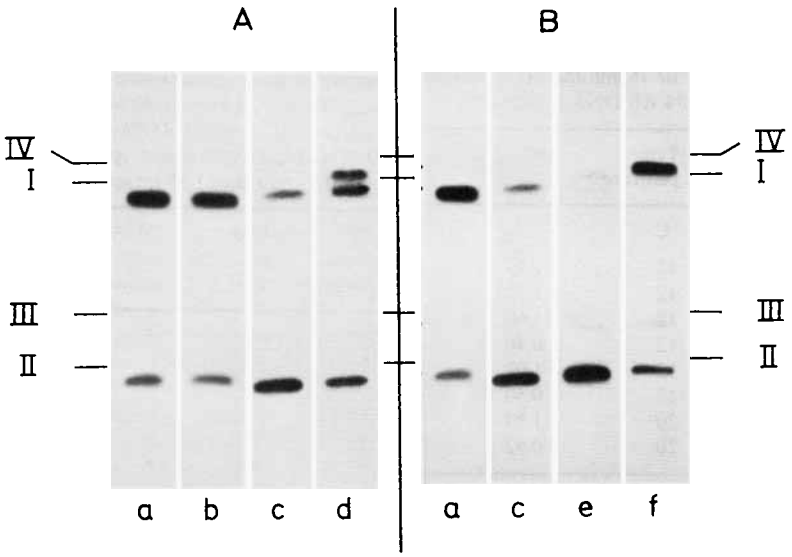

Fig. 1. *Repair of φX174 RF-I DNA containing apurinic sites; analysis by gel electrophoresis.* There was no alkaline cycle of denaturation so that apurinic sites not associated with nicks are not detected. The gels contained ethidium bromide and were revealed by fluorography. The positions of the four RFs (I, II, III and IV) are indicated. (A) Substrate φX174 depurinated RF DNA (a); after incubation with T4 ligase (b); after incubation with the chromatin AP endodeoxyribonuclease (c); the depurinated substrate was treated with the chromatin AP endodeoxyribonuclease and, after inactivation of this enzyme, with T4 ligase (d). (B) Substrate φX174 depurinated RF DNA (a); after incubation with the chromatin AP endodeoxyribonuclease (c); the depurinated substrate was treated successively with the chromatin AP endodeoxyribonuclease and 5′-3′-exonuclease, these enzymes were inactivated and the gapped molecules incubated either with T4 ligase alone (e) or with DNA polymerase β and T4 ligase (f)

Coming back to Table 2 and the nitrocellulose filter assay, one can see that a treatment with the chromatin 5′-3′ exonuclease after the AP endodeoxyribonuclease, in spite of a subsequent incubation with ligase, rather increased the sum of breaks and apurinic sites; the nicks were converted into gaps. When, however, DNA polymerase β was present together with ligase, there was a dramatic decrease of the sum of breaks and apurinic sites. With respect to the depurinated substrate, the reduction amounted to an average of about 0.8 break and apurinic site; if it is remembered that this substrate contained an average of 0.42 break and 0.90 apurinic site per molecule, the conclusion is that apurinic sites have been excised and the resulting gap repaired by the combined actions of DNA polymerase β and T4 ligase (in the second experiment, the reduction was an average of about 1.0 break and apurinic site whereas the depurinated substrate had an average of 0.47 break and 1.33 apurinic sites per molecule).

Fig. 1B illustrates the same experiment. Lanes a and c are the same as lanes a and c of Fig. 1A; for lanes e and f, the substrate was moreover treated with the 5′-3′ exonuclease and the gapped substrate incubated with ligase alone (lane e) or with DNA polymerase β and ligase (lane f). Ligase alone had little action on the gapped substrate whereas the appearance of an important RF-IV band when DNA polymerase β was also present indicates that repair had occurred. Since the amount of RF-II left in lane f is smaller than the amount resulting from the AP endodeoxyribonuclease treatment, it can be concluded that gaps produced where there were apurinic sites were refilled and sealed. It cannot be proved that the apurinic sites have disappeared from the repaired DNA since there was no alkaline treatment before the electrophoresis; but the proof of the disappearance of the alkali-labile sites was given with the nitrocellulose filtration technique.

The excision of apurinic sites was also observed when the AP endodeoxyribonuclease was omitted (Table 2). This was due to the presence, in the 5′-3′ exonuclease preparation, of an AP endodeoxyribonuclease activity which was easily demonstrated using φX174 depurinated RF-I DNA and gel electrophoresis. We do not know yet whether this AP endodeoxyribonuclease is an intrinsic property of the 5′-3′ exonuclease or if it is a contamination.

DISCUSSION

Three bacterial enzymes were able to repair *in vitro* DNA containing apurinic sites: endonuclease VI, DNA polymerase I and ligase [1]. The apurinic site was excised in a dinucleotide or trinucleotide by the 5′-3′ exonuclease activity of DNA polymerase I [2, 3].

We present here the successful repair of DNA containing apurinic sites by enzymes extracted from rat liver chromatin with one exception: we used T4 ligase since we did not have enough chromatin ligase.

DNA polymerase β was chosen for the polymerisation step since it is likely that the eukaryotic DNA polymerase is most often involved in DNA repair. Our preparation of DNA polymerase β did not contain any exonuclease activity; an exonuclease was thus needed for the excision step.

Since the chromatin AP endodeoxyribonuclease used hydrolyzed the phosphodiester bond 5′ to the apurinic site, the question arose whether chromatin contained a 5′-3′ exonuclease capable of starting from the nick introduced by the endonuclease and of excising the apurinic site. An exonuclease extracted from rat liver chromatin which, like DNase IV described by Lindahl et al. [9], degrades nicked strands of double-stranded DNA in the 5′-3′ direction (Zocchi, unpublished) was tried. To check that the apurinic sites had been excised by this exonuclease, the gaps were filled with DNA polymerase β and the new pieces ligated. The disappearance of breaks and alkali-labile sites showed that excision of apurinic sites had occurred.

Since a ligase was also found in rat liver chromatin (Bricteux, unpublished), the successful outcome of our repair experiment *in vitro* means that rat liver chromatin possesses all the necessary enzymes to repair DNA with apurinic sites by the excision pathway. We cannot tell however if the repair proceeds in the living cell as it did in the reconstituted system *in vitro*.

Two observations made during the course of this research must also be commented upon. Firstly, the chromatin 5′-3′ exonuclease had an AP endodeoxynuclease activity. It is not yet known whether it is an intrinsic property of the enzyme or whether it is a contaminant. This AP endodeoxyribonuclease very probably also cuts 5′ to the apurinic site since addition of the other AP endodeoxyribonuclease which certainly cuts on the 5′ side was not needed to observe the excision of the apurinic site. It must be recalled that Bose et al. [4] had a 5′-3′ exonuclease associated with the AP endodeoxyribonuclease, prepared from lymphoblast total cellular extract, which they used in their repair experiment.

Secondly, our results indicate that T4 ligase is able to reseal the nick introduced in the phosphodiester bond immediately neighbouring the apurinic site on its 5′ side; it must be emphasized that the 5′-phosphate limiting the nick belongs to a deoxyribose without a base. Indeed a formation of RF-IV dependent on a previous treatment of the depurinated RF-I DNA by the AP endodeoxyribonuclease was shown by gel electrophoresis (Fig. 1A); but the alkali-labile apurinic sites had not disappeared since the sealing could not be demonstrated after an alkaline cycle of denaturation by nitrocellulose filtration (Table 2).

Gossard and Verly [3] raised the question of the utility of the exonuclease III activity of *Escherichia coli* endonuclease VI in the repair of DNA with AP sites; they suggested an antiligase action: enlargement of the incision into a gap would prevent a premature closure by ligase leading to abortion of the repair. The results shown here give support to their hypothesis. On the other hand, a similar mechanism might operate in mammalian cells if the AP endodeoxyribonuclease involved in the repair of AP sites *in vivo* is the one associated with the 5′-3′ exonuclease. The difference would be that, in bacteria, formation of the gap results from exonuclease III degradation in the 3′-5′ direction which leaves the AP site in the DNA, whereas, in chromatin, the gap would be formed at the same time as the excision of the AP site by degradation in the 5′-3′ direction.

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