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T4 DNA ligase can seal a nick in double-stranded DNA limited by a 5'-phosphorylated base-free deoxyribose residue

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Summary : The 5' AP endodeoxyribonucleases hydrolyze the phosphodiester bond 5' to AP (apurinic or apyrimidinic) sites in double-stranded DNA leaving 3'-OH and 5'-phosphate ends. These nicks are sealed by T4 DNA ligase although the 5'-phosphate end belongs to a base-free deoxyribose.

INTRODUCTION

The 5'AP endodeoxyribonucleases incise the phosphodiester bond 5' to AP sites in double-stranded DNA leaving 3'-OH and 5'-phosphate ends [1,2,3,4]. We show in this paper that T4 DNA ligase can reseal these incisions leading to the reappearance of intact AP sites. The demonstration was carried out using widely different techniques with two different DNAs : the circular RF DNA of phage ϕX174 and the linear double-stranded DNA of phage T7.

THE ϕX174 RF DNA MODEL.

Tritium-labelled RF-I DNA of phage ϕX174, with a specific radioactivity of 650,000 dis.min^{-1}.μg^{-1}, was prepared as described in [5]. This [^{3}H]DNA was submitted to pancreatic deoxyribonuclease I to obtain nicked [^{3}H]DNA (about 1 nick per RF molecule), or incubated 60 min at pH 4.0 and 37° to obtain depurinated [^{3}H]DNA (about 1 AP site per RF molecule) [5].

Agarose gel electrophoresis in the presence of ethidium bromide followed by radioactivity determination of the separated bands was used to determine the average number n of strand breaks per RF molecule : (RF-I + RF-IV)/ΣRF = e^{-n}. To determine the average number n" of lesions (strand breaks + intact AP sites), the DNA was incubated at pH 11.7 and, after lowering the pH to

Table I : Action of T4 DNA ligase on different kinds of nicks in ϕX174 RF DNA.

exp	DNase	depur	AP endo	lig	breaks n	intact AP sites n'	total lesions n"
A	-			-	0.42		
	-			+	0.35		
	+			-	1.43		
	+			+	0.40		
B		+	-	-	0.42	0.80	1.22
		+	-	+			1.09
		+	+	+			1.17

A.- ϕX174 RF-I [^{3}H]DNA (650,000 dis.min^{-1}.µg^{-1}) has been prepared and kept 4 months at 4° in 0.15 M NaCl, 15 mM Na citrate, 0.1 mM EDTA, pH 7.0, 0.1 % benzyl alcohol. The solution was dialyzed against 50 mM Tris.HCl, 10 mM $MgCl_2$, pH 7.5, and 400 µl containing 24 µg of RF [^{3}H]DNA were incubated with 0.001 unit of DNase I for 12 min at 30°; the reaction was stopped with 100 µl 0.2 M EDTA, the proteins extracted with phenol, and the solution dialyzed against 5 mM NaCl, 1.5 mM Na citrate, 1 mM EDTA, pH 7.0. To 60 µl of the dialyzed solution were added 20 µl 1.6 mM ATP, 200 mM EPPS (4-[2-hydroxyethyl]-piperazine-1-propanesulfonic acid), 60 mM $MgCl_2$, 4 mM dithiothreitol, 0.16 % bovine serum albumin, pH 8.0, containing 1.2 units of T4 ligase; after 75 min at 20°, the reaction was stopped with EDTA to 20 mM final concentration. Sodium dodecylsulfate was added to 0.1 % final concentration and the solution heated at 65° for 5 min. After an alkalinization-neutralization cycle, the solution was filtered on nitrocellulose. Since there was no intact AP sites, n" = n, the average number of strand breaks per RF molecule.

B.- To 1 volume of RF [^{3}H]DNA (120 µg/ml) was added 1 volume of 0.2 M Na acetate buffer so that the final pH was 4.0; after 60 min at 37°, the pH was raised with 1 volume of 1 M K phosphate, pH 7.0, and the solution dialyzed against 5 mM NaCl, 1.5 mM Na citrate, 1 mM EDTA, pH 7.0. The depurinated [^{3}H]DNA (4 µg) and 250 units of AP endodeoxyribonuclease from rat-liver chromatin [4] in 130 µl of buffer A (50 mM EPPS, 15 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 0.04 % bovine serum albumin, pH 8.0) were incubated at 37° for 15 min; the reaction was stopped by heating 5 min at 65°. T4 ligase (2 units) in 10 µl buffer A containing 5.6 mM ATP were then added; the incubation and subsequent treatment were carried out as above, as was the assay on nitrocellulose filter after the alkalinization-neutralization cycle. The result here is n", the sum of strand breaks and intact AP sites per RF molecule. The number n of strand breaks was determined by gel electrophoresis.

DNase = pancreatic deoxyribonuclease I; depur = depurination at pH 4.0; AP endo = AP endodeoxyribonuclease; lig = ligase. In the two experiments, controls were carried out without one or the two enzymes. The error on n values is less than ± 0.1.

7.0, the solution was poured on a nitrocellulose filter which retained the irreversibly denatured DNA (RF-II and RF-III); if F is the fraction of the total radioactivity adsorbed on the filter : $1 - F = e^{-n''}$. The average number of intact AP sites (i.e. AP sites not associated with breaks) per RF molecule, $n' = n'' - n$ [5]. When there is no intact AP sites, either method can be used to determine the number of strand breaks since then : $n'' = n$; using such a DNA, we have shown the excellent agreement between the two methods.

On keeping untreated [^{3}H]DNA solution (120 µg/ml) at 4°, strand breaks accumulate due to radiolysis; incubation with T4 DNA ligase (BRL) was unable to mend these breaks whereas additional breaks introduced by deoxyribonuclease I were quantitatively repaired (Table I; exp. A).

Treatment of the depurinated [^{3}H]DNA with the chromatin 5' AP endodeoxyribonuclease from rat liver [4] resulted in the formation of breaks near most of the AP sites as measured after gel electrophoresis. After heat-inactivation of the AP endodeoxyribonuclease, an incubation with T4 DNA ligase resulted in no change of the total DNA lesions determined by nitrocellulose filtration after an alkalinization-neutralization cycle (Table I, exp. B). The same experiment is illustrated in the gels presented in figure 1. Lane 1 presents the depurinated [^{3}H]DNA; the RF-II molecules were mostly the result of radiolysis since an incubation with T4 DNA ligase led to no change (lane 2). On the other hand, incubation with the AP endodeoxyribonuclease transformed many RF-I molecules into RF-II (lane 3); this was the result of incisions near the AP sites. A subsequent treatment with T4 DNA ligase decreased the importance of the RF-II band and gave rise to an RF-IV band (lane 4). The RF-IV molecules (circular, relaxed but without strand breaks) were due to resealing of RF-II molecules; but these RF-IV molecules likely contained alkali-labile sites since the repair was not observed by the nitrocellulose filtration assay (Table I, exp. B).

The conclusion seems to be that RF-I molecules containing intact AP sites were incised, by the AP endodeoxyribonuclease, to give RF-II molecules which were transformed, by T4 DNA ligase, into RF-IV molecules where the intact AP sites had reappeared.

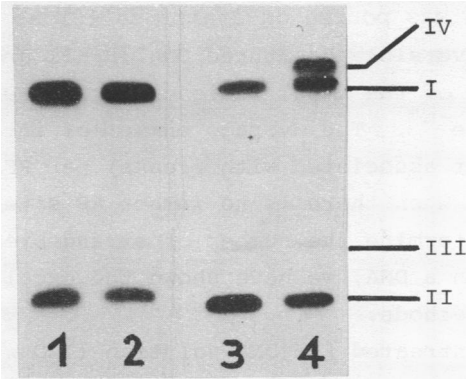

Figure 1 : Sealing by T4 DNA ligase of incisions made by a 5' AP endodeoxyribonuclease in depurinated ϕX174 RF [^{3}H]DNA.

Gels containing ethidium bromide (1.5 µg/ml) revealed by fluorography [6]. The electrophoresis was carried out from bottom (-) to top (+); the positions of the four RF DNAs are indicated.

Depurinated ϕX174 RF [^{3}H]DNA (lane 1); after treatment with T4 DNA ligase (lane 2); after incubation with the chromatin AP endodeoxyribonuclease (lane 3); after incubation with the chromatin AP endodeoxyribonuclease, heat-inactivation of this enzyme, and treatment with T4 DNA ligase (lane 4).

THE T7 DNA MODEL.

Tritium-labelled double-stranded DNA of phage T7, with a specific radioactivity of 329,000 dis.min^{-1}.µg^{-1}, was prepared as described in [7]. It was acid-depurinated to have about 5 AP sites per DNA strand (12,600,000 daltons). To determine the average numbers of breaks and intact AP sites per strand, the DNA was denatured with formamide at neutral pH which left the AP sites intact, or with NaOH followed by neutralization which placed a break near each AP site. The separated strands were centrifuged on neutral sucrose gradients which were fractionated for radioactivity measurements; the number of breaks was calculated from the sedimentation profile with a computer. After formamide denaturation, the breaks were those already present before denaturation; after NaOH treatment, the result gave the sum of preexisting breaks and those added near AP sites. The difference is the average number of intact AP sites per strand in the DNA before denaturation [8].

Two experiments were carried out (Table II). Since they lead

Table II : Action of T4 DNA ligase on incisions made by a 5' AP endodeoxyribonuclease near AP sites in double-stranded T7 [^{3}H]DNA.

exp	AP endo	lig	breaks		intact AP sites
			NaOH n"	formamide n	n'
I	-	-	6.46	1.59	4.87
	+	-	6.68	6.06	0.62
	+	+	5.48	2.44	3.04
II	-	-	4.46	1.30	3.16
	+	-	4.36	4.62	0.00
	+	+	4.14	1.74	2.40

To 60 µl 5 mM NaCl, 1 mM Na citrate, 0.1 mM EDTA, pH 8.0, containing 8.5 µg of depurinated [^{3}H]DNA, were added 25 µl 200 mM EPPS, 60 mM $MgCl_2$, 8 mM dithiothreitol, 0.4 mM EDTA, pH 8.0, and 10 µl 20 mM Tris.HCl, 1 mM EDTA, 0.2 mM dithiothreitol, 0.04 % bovine serum albumin, pH 8.0, containing 230 units of AP endodeoxyribonuclease from rat-liver chromatin, and the incubation was carried out for 15 min at 37°. The enzyme was inactivated by a 5-min heating at 65°. Were then added 25 µl 50 mM EPPS, 15 mM $MgCl_2$, 1 mM dithiothreitol, 0.04 % bovine serum albumin, pH 8.0, containing 7.2 units of T4 DNA ligase and ATP to have a 0.4 mM final concentration. After 2 h at 15°, the reaction was stopped by addition of 20 µl 0.2 M EDTA, pH 8.0, and 20 µl 1 % sodium dodecylsulfate, followed by a 2-min heating at 65°. Controls without one or the two enzymes were carried out in parallel.

The proteins were extracted with phenol and the samples divided in two for denaturation either with formamide at pH 7.0, or with NaOH followed by neutralization. The denatured DNAs were then analyzed by sedimentation on neutral sucrose gradients to determine the average number of breaks per strand [8].

AP endo, lig, n, n' and n" : see Table I. The error on n values is less than ± 0.1.

to the same conclusion, only experiment I will be commented. The depurinated [^{3}H]DNA contained an average of 1.59 breaks and 4.87 intact AP sites per strand. After treatment with the chromatin AP endodeoxyribonuclease, the number of intact AP sites was reduced to 0.62 per strand; on the other hand, there was no significant increase of the number of lesions (breaks + intact AP sites) showing that the endonuclease activity was specific for AP sites. After heat inactivation of the AP endodeoxyribonuclease, an incubation with T4 DNA ligase raised the number of intact AP sites from 0.62 to 3.04 per strand. The reappearance of intact AP

sites can be due only to the sealing by T4 DNA ligase of the incisions made by the AP endodeoxyribonuclease.

DISCUSSION.

The chromatin AP endodeoxyribonuclease from rat liver, acting on double-stranded DNA, hydrolyzes the phosphodiester bond which is the immediate neighbour of AP sites, on their 5' side, leaving 3'-OH and 5'-phosphate ends [4]. There is thus a 5'-phosphorylated deoxyribose residue without base at the 3' border of the nick. The question was whether a DNA ligase could seal such a peculiar break. The results of the experiments presented in this paper, using ϕX174 RF DNA or T7 DNA, show that T4 DNA ligase does indeed seal such a nick. It is possible that the sealing can also be performed by other DNA ligases.

Two kinds of AP endodeoxyribonucleases seem to exist : those nicking 5' and those nicking 3' to the AP sites [9]. All the 5' AP endodeoxyribonucleases do hydrolyze the same bond as the enzyme from rat-liver chromatin [1,2,3,4]; on the other hand, the 3' AP endodeoxyribonucleases need the help of a 5' AP endodeoxyribonuclease to participate in the repair of AP sites [9]. DNA ligase might thus interfere with the first step of AP site repair [2] unless some mechanism exists to prevent a premature ligation.

Endonuclease VI, the main AP endodeoxyribonuclease of Escherichia coli, is the same protein as exonuclease III [10,2] : incision 5' to the AP site is followed by an exonucleolytic degradation in the 3'-5' direction transforming the nick into a gap while leaving the AP site in DNA. Thus, as we already supposed several years ago [2], the biological function of the exonuclease III activity of endonuclease VI might well be that of an anti-ligase.

Such a mechanism does not seem to be present in eukaryotic cells where the chromatin AP endodeoxyribonuclease has no exonucleolytic activity [4], but the complexity of DNA repair in such a highly organized structure as chromatin might well order the steps of the repair so that the incision near the AP site cannot be immediately followed by ligation.

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