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Chromatin 3'-phosphatase/5'-OH kinase cannot transfer phosphate from 3' to 5' across a strand nick in DNA

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Summary : Rat liver chromatin contains a 3'-phosphatase/5'-OH kinase which may be involved in the repair of DNA strand breaks limited by 3'-phosphate/5'-OH ends. In order to determine whether the phosphate group can be transferred directly from the 3' to the 5' position, a polynucleotide duplex was synthesized between poly(dA) and oligo(dT) segments which had 3'-[^{32}P]phosphate and 5'-OH ends. The oligo(dT) segments were separated by simple nicks as shown by the ability of T4 DNA ligase to seal the nick after the 3'-phosphate was removed by a phosphatase and the 5' end was phosphorylated with a kinase. The chromatin 3'-phosphatase/5'-OH kinase was unable to transfer phosphate directly from the 3' to the 5' end of the oligo(dT) segments in the original duplex; ATP was needed to phosphorylate the 5'-OH end. It is concluded that the chromatin 3'-phosphatase/5'-OH kinase is unable to convert a 3'-phosphate/5'-OH nick which cannot be repaired by DNA ligase directly into a 3'-OH/5'-phosphate nick which can be repaired by DNA ligase; the chromatin enzyme rather acts in two steps : hydrolysis of the 3'-phosphate followed by ATP-mediated phosphorylation of the 5'-OH end.

INTRODUCTION

We have found [1] and purified to near homogeneity an enzyme from rat-liver chromatin that specifically hydrolyzes 3'-phosphates in DNA; the protein is also a polynucleotide 5'-OH kinase. Pheiffer and Zimmerman [2] found that a preparation of DNA kinase, purified from rat-liver nuclei, contained a 3' phosphatase activity. T4 polynucleotide 5'-OH kinase has also a 3' phosphatase activity [3]. The association of these two activities on the same protein in organisms as far apart as T4 phage and rat suggests the possibility that they may participate to a concerted repair mechanism.

3'-phosphate ends are found in irradiated DNA [4] where they are usually separated from 5'-phosphate ends by a one-nucleotide gap [5]. Simple nicks limited by 3'-phosphate and 5'-

OH ends are less common; they may exist in limited numbers in irradiated DNA or result from an aborted reaction catalyzed by an eukaryotic topoisomerase which cleaves the phosphodiester bonds in 3'-phosphates that normally attach to tyrosine residues of the enzyme, and 5'-OH [6].

The problem that we tried to solve in this work is whether 3'-phosphate/5'-OH nicks which DNA ligase cannot seal can be transformed in one step by the chromatin 3'-phosphatase/5'-OH kinase into 3'-OH/5'-phosphate nicks that DNA ligase can seal. This could occur if the enzyme could transfer the phosphate directly from 3' to the 5' end (figure 1).

MATERIALS and METHODS

Materials :

Chromatin 3'-phosphatase/5'-OH kinase was prepared from rat liver [1 and unpublished]. Deoxynucleotidyl terminal transferase, T4 polynucleotide kinase, T4 DNA ligase, deoxynucleosides triphosphates and ATP were purchased from Boehringer; alkaline phosphatase from Sigma; $(dT)_8$ and $(dA)_8$ from PL-Biochemicals; Sephadex G-25 and Blue Sepharose from Pharmacia. The radioactive nucleotides came from Amersham.

Buffer A : 50 mM Na succinate, 10 mM $MgCl_2$, 1.3 mM 2-mercaptoethanol, bovine serum albumin (0.03 mg/ml), pH 5.5.

Buffer B : 0.5 M sodium cacodylate, 25 mM $MgCl_2$, 0.85 mM $ZnCl_2$, 2 mM 2-mercaptoethanol, bovine serum albumin (5 mg/ml), pH 7.0.

Acid-insoluble radioactivity :

Aliquots of the polymerization mixtures were brought to

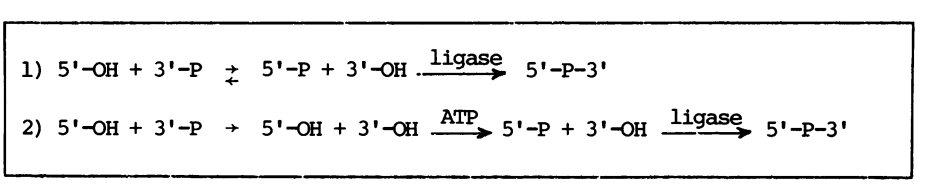

Figure 1 : Two models for the repair of 3'-phosphate/5'-OH nicks

1) phosphate transfer from 3' to 5' end of the nick; even if, when isolated, the first step might come to an equilibrium, it would go to completion when coupled with the ligation step.

2) hydrolysis of 3'-phosphate followed by ATP-mediated phosphorylation of 5'-OH.

100 µl with water, and then 100 µl 14 mM $Na_2P_2O_7$-bovine serum albumin (0.5 mg/ml) and 200 µl 10 % $HClO_4$ were added. After 10 min at 0°C, the tubes were centrifuged for 10 min at 12,000 x g. The supernatants were discarded and the pellets were dissolved in 100 µl 0.1 M NaOH and reprecipitated with 400 µl 5 % $HClO_4$. Finally, these precipitates, collected by centrifugation, were redissolved in 1 ml 2 M NH_4OH for radioactivity determination.

Inorganic phosphate radioactivity :

To the tubes containing the reaction solution (100 µl maximum), cooled on ice, were added : 100 µl 25 mM $Na_4P_2O_7$, 20 mM KH_2PO_4, bovine serum albumin (0.5 mg/ml); 400 µl 10 % $HClO_4$; and 200 µl activated charcoal (20 % in water). The mixtures were shaken a few minutes and, after 10 more minutes at 0°C, were centrifuged for 10 min at 12,000 x g. The radioactivity of the supernatants, which contained the inorganic phosphate, was measured.

Treatment with T4 polynucleotide kinase or with chromatin 3'-phosphatase/5'-OH kinase :

Reaction mixture consisted of : 50 µl poly(dA)/poly(dT) (about 0.2 µg) in 10 mM Tris.HCl, pH 7.5; 12 µl 100 µM ATP (^{32}P-labelled in γ position [6 µCi] in some experiments) in water; 10 µl T4 polynucleotide kinase (4.5 units) or chromatin 3'-phosphatase/5'-OH kinase in buffer A; and 37.5 µl of 3 x concentrated buffer A. After 1 h at 37°C, another 10-µl enzyme solution was added and the incubation was carried out for another 1 h.

Treatment with T4 DNA ligase :

32 µl 210 mM Tris.HCl, 30 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, pH 7.60, 60 µl poly(dA)/poly(dT) (about 0.25 µg) in 10 mM Tris.HCl, pH 7.5, 1 µl T4 DNA ligase (1 unit) solution, were incubated 16 h at 16°C.

EXPERIMENTS and RESULTS

1) Preparation of a double-stranded polydeoxynucleotide substrate containing 3'-phosphate/5'-OH nicks :

1.1 - Synthesis of poly(dA) : A mixture of 5.05 nmol $(dA)_8$, 1125 nmol [^{3}H]dATP (28 µCi), 400 units of deoxynucleotidyl terminal transferase, 600 µl buffer B, completed to 2 ml with water, was incubated at 37°C for 6 h. An aliquot showed that 85 % of

the radioactivity had become acid-insoluble. The proteins were eliminated by filtration through a Blue Sepharose column equilibrated with 10 mM NH_4HCO_3. The mononucleotides were eliminated by filtration through a Sephadex G-25 column; the fractions corresponding to the poly(dA) peak, detected by absorbance at 260 nm and radioactivity measurements, were pooled. The average length of the [^{3}H]poly(dA) molecules was : $\frac{1125 \times 0.85}{5.05} + 8 = 197$ nucleotides.

1.2 - Synthesis of poly(dT,dA) : A mixture of 5.5 nmol $(dT)_8$, 2400 nmol [^{3}H]dTTP (40 µCi), 40 nmol [α-^{32}P]dATP (1 µCi), 250 units deoxynucleotidyl terminal transferase, 600 µl 0.2 M potassium cacodylate, 4 mM $CoCl_2$, 0.4 mM $ZnCl_2$, bovine serum albumin (3 mg/ml), pH 7.4, 0.2 mM β-mercaptoethanol (added just prior utilization), completed to 1.5 ml with water, was incubated at 37°C for 16 h; 56 % of [^{3}H] and 92 % of [^{32}P] radioactivities had become acid-insoluble. Proteins and mononucleotides were eliminated as described previously (1.1) except that elution was carried out with 10 mM Tris.HCl, pH 7.5.

The average elongation of $(dT)_8$ is $\frac{(2400 \times 0.56) + (40 \times 0.92)}{5.5}$ = 251 nucleotides, and the T/A ratio $\frac{2400 \times 0.56}{40 \times 0.92} = 36$.

1.3 - Depurination of poly(dT,dA) and fragmentation by β elimination : The poly(dT,dA) solution was made 10 mM with HCl and heated 5 min at 100°C to hydrolyze away the A's (figure 2); it was then neutralized with NaOH.

The extent of depurination was measured on an aliquot after addition of NaOH up to 0.2 M and a 30-min heating at 65°C (to fragment the polynucleotide and induce βδ elimination of the apurinic sites), HCl neutralization, exhaustive treatment with alkaline phosphatase and determination of the inorganic phosphate radioactivity. In a pilot preparation, the depurination was 100 %, but, when the scale was increased 10 times, the depurination was only 59 %.

The solution of polynucleotides in which apurinic sites had replaced 59 % of the A's, was brought to pH 11.5 by addition of 1 M glycine.NaOH, pH 12, then incubated at 37°C. Fragmentation of the polynucleotide molecules at apurinic sites by β-elimination (figure 2) was followed by determination of the radioactivity soluble in 5 % $HClO_4$ at 0°C. When it was at a maximum

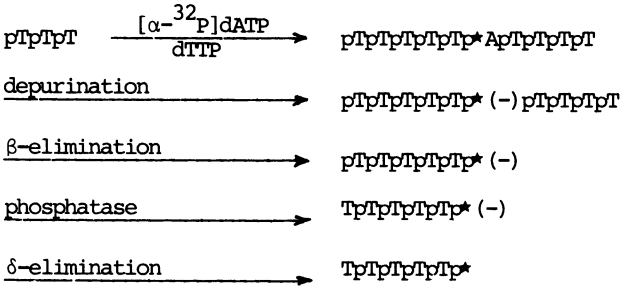

Figure 2 : Synthesis of oligo(dT) with 3'-[^{32}P]phosphate and 5'-OH ends.

and that, consequently, the fragmentation was complete, the 5'-phosphate-terminated pieces had an average length of : (37/0.59) - 1 = 62 nucleotides plus an apurinic site at the 3' end.

1.4 - Hydrolysis of the 5'-phosphate and δ-elimination of the apurinic site : The solution (1.23 ml) of depurinated and fragmented polynucleotide (19 µg) was brought to pH 9 and, after addition of 2.5 units of alkaline phosphatase, incubated at 37°C for 30 min (figure 2).

NaOH was subsequently added up to 0.2 M before heating at 65°C for 30 min to eliminate the apurinic sites (figure 2). After cooling, the solution was neutralized with HCl.

The last solution contained polynucleotides of 62-nucleotide average length with 5'-OH and 3'-[^{32}P]phosphate ends. This was checked on an aliquot : exhaustive alkaline phosphatase treatment released 44 % of the ^{32}P as inorganic phosphate; since we had only a 59 % depurination, this result means that all later steps had a yield near maximum.

1.5 - Hybridization : The solutions of poly(dA)$_{197}$ (30 nmol A) and poly(dT)$_{62}$ (33 nmol T) were mixed and warmed 7 h at 22°C. The slight excess of T's relative to A's, was believed to oblige the 3'-[^{32}P]phosphate end of a poly(dT) molecule to lie as close as possible to the 5'-OH end of the next molecule leaving a simple nick between them in the T strand of the duplex.

2) Checking the substrate

In this experiment, we used the poly(dT,dA) of the pilot preparation where the depurination was 100 % (see 1.3). A βδ

elimination performed in one step, followed by an exhaustive treatment with alkaline phosphatase, gave poly(dT) molecules, limited by 3'-OH and 5'-OH ends (the ^{32}P was completely lost), having an average length of 36 nucleotides.

Poly(dA)$_{197}$ and poly(dT)$_{36}$ were mixed in a T/A ratio of 1.1 and annealed. The hybrid (0.21 µg) was treated with T4 5'-OH kinase (4.5 units) and [γ-^{32}P]ATP, then the solution was filtered through a Sephadex G-25 column to eliminate the radioactive ATP and inorganic phosphate. In the hybrid, the fragments of the T strand then had 3'-OH and 5'-[^{32}P]phosphate ends. Treatment of an aliquot of this preparation by alkaline phosphatase released 96 % of the ^{32}P as inorganic phosphate.

The hybrid (16 ng) of poly(dA) and poly(dT) with 5'-[^{32}P] phosphate ends, was incubated with T4 DNA ligase and ATP. Subsequent exhaustive treatment with alkaline phosphatase released only 42 % of the ^{32}P as inorganic phosphate. The DNA ligase treatment prevented the release of $\frac{96 - 42}{96}$ = 56 % of the phosphate, meaning that 56 % of the 5'-phosphates of the poly(dT) fragments had been included in phosphodiester bonds.

We may conclude that many fragments of the T strand were contiguous; if a gap had remained between them, no phosphodiester linkage could have been formed by the DNA ligase.

3) Tentative transfer of phosphate from 3' to 5' ends across a nick using the 3'-phosphatase/5'-OH kinase of rat-liver chromatin

In this experiment, we used the hybrid prepared in (1.5) where the T strand fragments were limited by 5'-OH and 3'-[^{32}P] phosphate ends containing 44 % of the substrate ^{32}P.

When this hybrid (1.2 µg) was incubated with the chromatin 3'-phosphatase/5'-OH kinase, 37 % of the ^{32}P was released as inorganic phosphate whether or not 10 µM ATP was present. After filtration through a Sephadex G-25 column to eliminate the radioactive inorganic phosphate, a subsequent treatment with alkaline phosphatase did not release any more inorganic [^{32}P]phosphate (Table I); an incubation with T4 DNA ligase interposed between the chromatin enzyme and alkaline phosphatase treatments, obviously did not change the result. The conclusion is that the 3'-[^{32}P]phosphate ends of the substrate were completely hydrolyzed by the chromatin 3'-phosphatase/5'-OH kinase and that no

Table I : Evidence that the conversion of a 3'-phosphate/5'-OH into a 3'-OH/5'-phosphate nick in DNA by the chromatin 3'-phosphatase/5'-OH kinase is a two-step reaction.

[γ-^{32}P]ATP	DNA ligase	PO_4 released by phosphatase (%)
-	-	0
-	+	0
+	-	54
+	+	23

The hybrid constituted by poly(dA) and poly(dT) fragments with 3'-[^{32}P]phosphate and 5'-OH ends (see 1.5) was incubated with the chromatin 3'-phosphatase/5'-OH kinase, with or without [γ-^{32}P]ATP. After filtration through a Sephadex G-25 column to eliminate the small radioactive molecules, the modified hybrid was incubated or not with T4 DNA ligase, before being submitted to alkaline phosphatase; the amount of inorganic [^{32}P]phosphate released by this latter treatment is expressed as percents of the ^{32}P radioactivity of the modified hybrid substrate after the filtration step.

[^{32}P]phosphate was transfered from 3' to 5' ends.

The hybrid treatment with the chromatin 3'-phosphatase/5'-OH kinase was also carried out in the presence of 10 µM [γ-^{32}P] ATP, before filtration through a Sephadex G-25 column to eliminate the radioactive ATP and inorganic phosphate. The ^{32}P radioactivity of the hybrid is now that left after hydrolysis of the 3'-[^{32}P]phosphate ends by the 3' phosphatase activity of the chromatin enzyme and the [^{32}P]phosphate from [γ-^{32}P]ATP added to the 5' ends by the 5'-OH kinase activity of the chromatin enzyme. A subsequent treatment with alkaline phosphatase released 54 % of the substrate ^{32}P as inorganic phosphate; a preliminary treatment with T4 DNA ligase reduced this amount to 23 % (TableI). This control shows that, in the T strand of the hybrid, fragments were close enough so that, when their extremities had been changed to 3'-OH and 5'-phosphate, T4 DNA ligase could seal the interruption.

DISCUSSION

It is intriguing to find 3'-phosphatase and 5'-OH polynucleotide kinase activities within the same protein as well in the rat as in T4 phage-infected Escherichia coli. These two

activities are capable of converting a 3'-phosphate/5'-OH strand nick, unreparable by DNA ligase, into a reparable 3'-OH/5'-phosphate nick in double-stranded DNA. The question was whether the conversion occurred in one or two steps (figure 1). In the one-step model, the phosphate would be moved from 3' to 5' by a concerted mechanism mediated by the two activities so that, if ATP was needed, it was only as an allosteric effector. In the two-step model, the 3' phosphatase first hydrolyzes the 3'-phosphate, the phosphate being recovered as inorganic phosphate, and ATP supplies its γ-phosphate to phosphorylate the 5'-OH end.

The experiments presented here do not show any hint of the possibility for the phosphate to be transferred from 3' to 5' across a nick in a polydeoxynucleotide duplex. It thus seems that conversion of 3'-phosphate/5'-OH into 3'-OH/5'-phosphate nick by the chromatin 3'-phosphatase/5'-OH kinase is a two step process; the simultaneous presence of the two activities within the same protein might have kinetical advantages, otherwise it remains a complete mystery.

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