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The DNA 3′-phosphatase and 5′-hydroxyl kinase of rat liver chromatin

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1. INTRODUCTION

Some nicks, accidently produced in DNA, might be limited by 3′-phosphate and 5′-OH ends and not be suitable for ligase or polymerase activities. A 3′-phosphatase might thus be helpful to the cell whereas access of a 5′-phosphatase to nuclear DNA could only be injurious.

We have studied the action of chromatin proteins on double-strand DNA containing nicks limited by 3′-phosphate/5′-OH or 3′-OH/5′-phosphate ends, thus mimicking possible situations of the cell nuclear DNA. In agreement with the above prediction, chromatin proteins had only a 3′-phosphatase activity; this is in contrast with cytoplasmic proteins which hydrolyze both 3′-phosphate and 5′-phosphate ends.

T4 polynucleotide 5′-OH kinase has an associated 3′-phosphatase activity [1]. We have shown that chromatin likely possesses a protein with the same two activities: a 5′-OH kinase activity accompanies the 3′-phosphatase during its purification. This work was completed when the complementary observation was published [2]: the purified DNA kinase from rat liver contains a 3′-phosphatase activity.

2. MATERIALS AND METHODS

2.1. *Cytoplasmic and chromatin proteins*

We follow the method described in [3]. The rat liver homogenate is centrifuged 25 min at 20000 × g. The supernatant, after sonication, is centrifuged 30 min at 20000 × g, and the second supernatant is used as a solution of cytoplasmic proteins. The cell nuclei are isolated from the sediment of the first centrifugation and used to prepare chromatin. The chromatin is dissociated with heparin–Ultrogel (LKB) and the complex is extracted with 0.5 M KCl, 10 mM KH_2PO_4, 10 mM Tris–HCl (pH 8.0) [4].

2.2. *Preparation of 3′-phosphate/5′-OH and 3′-OH/5′-phosphate nicked [^32^P]DNA*

Escherichia coli B41 was cultivated in the presence of [^{32}P]phosphate and the [^{32}P]DNA was extracted as in [5].

To produce nicks limited by 3′-phosphate and 5′-OH ends, the [^{32}P]DNA was treated with micrococcal nuclease (Worthington) in 10 mM Tris–HCl, 1 mM $CaCl_2$ (pH 8.5) and the reaction was stopped by adding EGTA. This nicked DNA also contains a few small gaps because the nuclease has some exonucleolytic activity.

To produce nicks limited by 3′-OH and 5′-phosphate ends, the [^{32}P]DNA was treated with pancreatic DNase I (Boehringer) in 55 mM Tris–HCl, 5.5 mM $MgCl_2$ (pH 7.5) and the reaction is stopped by heating 15 min at 65°C.

Nick frequencies are calculated from the radioactivity soluble in 5% perchloric acid at 0°C.

2.3. *Assays for hydrolytic activities on 3′-phosphate and 5′-phosphate ends in double-stranded nicked [^{32}P]DNAs*

Glycine–NaOH (80 μl, 0.1 M), 1 mM $MgCl_2$

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(pH 8.0) 10 μl containing from 0.2–2 μg of [^{32}P]DNA nicked either with micrococcal nuclease (3′-phosphate ends) or pancreatic DNase I (5′-phosphate ends), and 10 μl protein solution were incubated at 37°C for 30 min. After addition of 0.4 ml 0.7 N trichloroacetic acid, 0.1 ml 20 mM $Na_4P_2O_7$, 25 mM KH_2PO_4, containing 50 μg bovine serum albumin, and 0.2 ml 20% Norit in water, the mixture was shaken for 5 min, centrifuged 10 min at 12000 × *g* before measuring the radioactivity of the supernatant which contains the liberated P_i.

2.4. *Polynucleotide 5′-OH kinase assay*

Calf thymus DNA (Sigma) was activated with micrococcal nuclease (about 35 nicks/1000 nucleotides). To 50 μl 50 mM Tris–HCl, 10 mM $MgCl_2$, 5 mM dithiothreitol (pH 7.6) containing 4 μg bovine serum albumin, were added 25 μl containing 30 μg of activated DNA and 10 μl 3 mM [γ-^{32}P]ATP (0.6 μCi). After 30 min at 37°C, 0.5 ml 1 N trichloroacetic acid is used to precipitate the DNA which is collected on Whatman GF/C filters. These filters are washed 5-times with 5 ml 0.5 N trichloroacetic acid, 2-times with 5 ml ethanol, and dried before measuring their radioactivity.

2.5. *Assay of priming activity for DNA polymerase I*

The incubation mixture contains: 50 μl 0.25 M glycine–NaOH, 35 mM $MgCl_2$, 3.7 mM 2-mercaptoethanol (pH 9.2); 25 μl water containing the 4 deoxynucleosides triphosphates (0.2 mM each; 9 μCi [^{3}H]dATP, Amersham); 4 μg DNA and 20 μl water containing 0.25 unit of the Klenow fragment of *E. coli* DNA polymerase I (Boehringer). After 30 min at 37°C, the reaction is stopped by adding 0.5 ml 1 N trichloroacetic acid and the radioactivity incorporated in DNA is measured as in section 2.4.

2.6. *Protein assay*

The method in [6] is followed using a standard of bovine serum albumin.

3. EXPERIMENTS AND RESULTS

3.1. *The chromatin 3′-phosphatase*

Chromatin proteins or cytoplasmic proteins were incubated with [^{32}P]DNA nicked by micrococcal nuclease (81 nicks/1000 nucleotides), [^{32}P]DNA nicked by pancreatic DNase I (91 nicks/1000 nucleotides), or untreated [^{32}P]DNA as in section 2.3. The results (table 1) were corrected for controls incubated without proteins.

Table 1

Hydrolysis of 3′-phosphates or 5′-phosphates limiting nicks in double-stranded DNA

DNA		Chromatin proteins		Cytoplasmic proteins		Theoretical
Description	cpm	cpm	%	cpm	%	%
Untreated	1700	0	0			0
3′-P	2056	199	9.7			8.1
5′-P	1720	12	0.7			9.1
Untreated	1336			8	0.6	0
3′-P	886			82	9.3	8.1
5′-P	871			435	50.0	9.1

[^{32}P]DNA untreated, or nicked with micrococcal nuclease (3′-P) or DNase I (5′-P), were incubated with chromatin or cytoplasmic proteins for 30 min at 37°C. The table gives the radioactivities of the initial substrate (cpm) and of the inorganic phosphate liberated at the end of incubation (cpm; % total). The theoretical percentage is calculated from the nick frequencies in the initial substrates, assuming that all phosphomonoesters have been hydrolyzed

The cytoplasmic proteins contain a non-specific phosphatase which hydrolyzes 5′-phosphate ends even better than 3′-phosphate ends. The result with [^{32}P]DNA nicked with pancreatic DNase I is high above the theoretical value calculated on the assumption that only the 5′-phosphate ends existing in the initial substrate are hydrolyzed. It can be explained if, as is likely, the cytoplasmic proteins contain nuclease activities that produce new phosphomonoesters sensitive to the phosphatase. A reason for not observing the same excess when [^{32}P]DNA nicked with micrococcal nuclease was used could be the following: the phosphatase is less active on 3′-phosphates than on 5′-phosphates and the appearance of new phosphomonoesters depends mostly on an exonucleolytic activity that needs 3′-OH ends.

The behaviour of the chromatin proteins is completely different. They hydrolyze 3′-phosphates, but have practically no effect on 5′-phosphates. The slight activity on 5′-phosphates can be totally suppressed with 0.5 mM ATP which has no effect on the 3′-phosphatase.

3.2. *The chromatin 3′-phosphatase makes a suitable primer for DNA polymerase of DNA treated with micrococcal nuclease*

Calf thymus DNA activated or not with micrococcal nuclease (25 nicks/1000 nucleotides when activated) was submitted or not to chromatin proteins before it was tested as primer for the polymerase activity of the Klenow fragment of *E. coli* DNA polymerase I. The results are in table 2.

Table 2

DNA priming activity and chromatin 3′-phosphatase

Micrococcal nuclease	Chromatin proteins	cpm
–	–	473
–	+	600
+	–	131
+	+	767

DNA nicked or not with micrococcal nuclease and subsequently treated or not with chromatin proteins, was used as template-primer for the Klenow fragment of DNA polymerase I in the presence of the 4 deoxyribonucleosides triphosphates, one of them being labelled (dATP). After incubation, the radioactivity incorporated into DNA was measured (cpm)

The untreated DNA had some primer activity that was slightly increased by a treatment with chromatin proteins (Δ = 127 cpm). Treatment with micrococcal nuclease decreased the primer activity of the DNA since 3′-phosphates have an inhibitory action on DNA polymerase I, but this primer activity was greatly enhanced by an additional treatment with chromatin proteins (Δ = 636 cpm). Most of this increase must be due to the 3′-phosphatase activity of the chromatin proteins which replaced 3′-phosphate ends by 3′-OH primers.

3.3. *Purification of the chromatin 3′-phosphatase*

Chromatin proteins prepared from the livers of 6 rats were dialyzed against 20 mM K phosphatase, 1 mM dithiothreitol, pH 6.5. After centrifugation, the supernatant was placed on a 7 × 1.6 cm column of phosphocellulose (Whatman), and the proteins eluted with a 0–0.6 M KCl linear gradient in the same buffer (fig.1a). Fractions 47–51 of the preceding chromatography were pooled and dialyzed against 20 mM imidazole–HCl, 1 mM dithiothreitol (pH 7.0) and placed on a 15 × 1 cm column of DEAE–Trisacryl (LKB), then eluted with 0.1 M NaCl in the same buffer (fig.1b).

The hydrolytic activities toward 3′-phosphates, 5′-phosphates, and 5′-OH DNA kinase were assayed on each fraction. Hydrolysis of 5′-phosphates could not be detected. The elution positions of 3′-phosphatase and 5′-OH DNA kinase were coincident in the two chromatographies. With the DEAE–Trisacryl, both activities were found in two peaks, one in the flow-through and one eluted with 0.1 M NaCl.

3.4. *Some properties of the chromatin 3′-phosphatase*

The chromatin 3′-phosphatase is inhibited by EDTA. Anions, such as phosphate, pyrophosphate or sulfate, which are known to inhibit the 5′-OH DNA kinase of rat liver [7], are without action on the activity of the chromatin 3′-phosphatase.

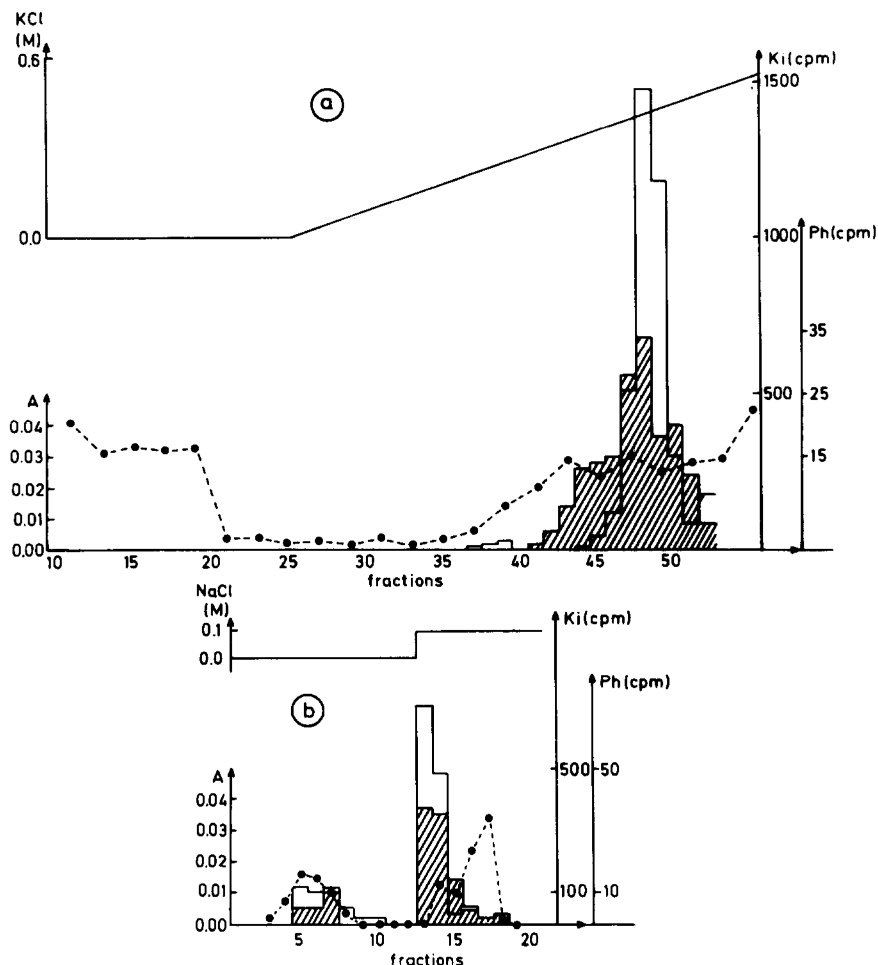

Fig.1. Purification of the chromatin 3′-phosphatase. (a) The dialyzed chromatin proteins in 58 ml 20 mM K-phosphate, 1 mM dithiothreitol (pH 6.5) (buffer A), were placed on a 7 × 1.6 cm phosphocellulose column equilibrated with buffer A. After washing with 35 ml buffer A, the proteins were eluted with 120 ml 0–0.6 M KCl linear gradient in buffer A. Fractions of 3.2 ml were collected. (b) Fractions 47–51 from the preceding chromatography were pooled and dialyzed against 20 mM imidazole–HCl, 1 mM dithiothreitol (pH 7.0) (buffer B). The sample (13 ml) was placed on a 15 × 1 cm DEAE–Trisacryl column equilibrated with buffer B. After washing with 20 ml of buffer B, the proteins were eluted with 0.1 M NaCl in buffer B. Fractions of 3.2 ml were collected. The continuous lines follow the KCl (part a) or NaCl (part b) concentration. Black dots and discontinuous lines give the absorbance A at 280 nm. Histograms refer to the enzyme activities: phosphate liberated by the 3′-phosphatase from [^{32}P]DNA nicked with micrococcal nuclease (shaded histogram; [Ph (cpm)] or ATP [γ-^{32}P]phosphate incorporated in DNA by the 5′-OH kinase [K_i (cpm)].

4. DISCUSSION

Chromatin proteins contain a 3′-phosphatase acting on nicks in double-stranded DNA. The hydrolytic activity on 5′-phosphate of these proteins is very low and can be entirely inhibited with a high concentration of ATP; it is suggested that, in absence of ATP, the 5′-OH DNA kinase could act on 5′-phosphates as a very slow hydrolase.

Chromatin proteins contain a 5′-OH DNA kinase which accompanies the 3′-phosphatase in two chromatographic steps. Fig.1a,b does not show a constant ratio of the two activities, but this is not to be expected since the assay was not done

in conditions where the amount of product was proportional to the amount of enzyme. It is thus possible that, as for the T4 polynucleotide 5′-OH kinase [1], the same chromatin protein has the two activities. It is also the conclusion reached independently in [2]. Nicks limited by 3′-phosphate and 5′-OH ends are not suitable for ligation; it is surprising that the two activities needed to change them into nicks limited by 3′-OH and 5′-phosphate ends seem to belong to the same protein. The obvious question is whether the phosphate is transferred from the 3′ to the 5′ position without ever becoming an exchangeable P_i and what could be the role of ATP in such a transfer. The inhibition of the kinase but not of the phosphatase by anions such as phosphate, pyrophosphate or sulfate, suggests that the two active sites are independent even if the two reactions are concerted.

Since the two activities were demonstrated on nicked double-stranded DNA, it seems that this chromatin protein might have an important function in nuclear DNA repair when a nick or a gap is limited by a 3′-phosphate. We have shown that the chromatin 3′-phosphatase can transform the inhibitory 3′-phosphate into a 3′-OH suitable to prime a DNA polymerase activity. If the lesion is a nick rather than a gap, as we explained above, the concerted action of the two activities of the chromatin protein might permit the ligation that completes the repair.

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