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Deoxyribonuclease IV from rat liver chromatin and the excision of apurinic sites from depurinated DNA

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Deoxyribonuclease IV, a 5′–3′ exonuclease degrading double-stranded DNA from intra-strand nicks, has been purified from the chromatin of rat liver cells. The enzyme, which has an M_r of 58000, excises the apurinic (AP) sites from a depurinated DNA nicked 5′ to these AP sites with the chromatin AP endonuclease. The excision is not the result of hydrolysis of the phosphodiester bond 3′ to the AP sites since the excision product does not behave as deoxyribose 5-phosphate but as its 2,3-unsaturated derivative. This result suggests that, to remove the AP sites from the DNA nicked by an AP endonuclease, the chromatin deoxyribonuclease IV rather acts as a catalyst of β-elimination.

Deoxyribonuclease (DNAase) IV is an exonuclease degrading double-stranded DNA from nicks in the 5′–3′ direction that was found in rabbit bone marrow by Lindahl *et al.* (1969). Lindahl (1971) moreover demonstrated that DNAase IV was able to remove pyrimidine dimers from u.v.-irradiated DNA.

We have found a similar activity in rat liver; the enzyme is mainly located in the cell nuclei and, particularly, in the non-histone proteins of chromatin. We also show in the present work that the chromatin DNAase IV is able to remove an AP site from DNA previously treated with a 5′ AP endonuclease and we describe the mechanism of this excision.

Materials and methods

Substrates

[^{3}H]DNA was prepared from *Escherichia coli* B41 cultivated in a minimal medium containing 1 μCi of [*methyl*-^{3}H]thymidine/ml (5 Ci/mmol; The Radiochemical Centre, Amersham).

[^{14}C]DNA was prepared from a thymineless mutant of *E. coli* [A1 *thy*$^-$ (Verly *et al.*, 1974)] cultivated in a minimal medium containing, per ml, 50 nCi in 10 μg of [*methyl*-^{14}C]thymidine (The Radiochemical Centre, Amersham).

DNA labelled in deoxyribose and adenine residues was prepared from *E. coli* grown in the presence of 0.5 μCi of [2,8,5′-^{3}H]adenosine/ml (37 Ci/mmol; The Radiochemical Centre, Amersham).

Poly(dA-[*methyl*-^{3}H]dT) was bought from Miles (13 Ci/mol of P) and calf thymus DNA from Sigma.

Non-histone proteins from rat liver chromatin

All the solutions used for the preparation of chromatin and its dissociation contained 0.5 mM-phenylmethanesulphonyl fluoride. The nuclei were isolated from the liver cells and the chromatin was prepared according to Thibodeau & Verly (1980). The chromatin was dissociated with heparin–Ultrogel and the non-histone proteins were eluted with 10 mM-potassium phosphate/0.5 M-KCl/10 mM-Tris/HCl, pH 8.0 (Renard & Verly, 1980).

Enzymes

The AP endonuclease was prepared from the non-histone proteins of rat liver chromatin. The enzyme is specific for AP sites and has no exonuclease activity; it hydrolyses the phosphodiester linkage neighbouring the AP site on its 5′ side, leaving 3′-hydroxy and 5′-phosphate ends (César & Verly, 1983). One enzyme unit hydrolyses 1 pmol of phosphoester bonds/min at 37°C.

Alkaline phosphatase, the Klenow fragment of *E. coli* DNA polymerase I and pancreatic deoxyribonuclease were bought from Boehringer, and T4 phage polynucleotide 5′-hydroxykinase from P.-L. Biochemicals.

Abbreviations used: AP, apurinic or apyrimidinic; DNAase, deoxyribonuclease; SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

Treatments of DNA or synthetic polynucleotide

Acid depurination. To 1 vol. of SSC, pH 7.0, containing 40–200 μg of DNA, labelled or not, was added 1 vol. of 0.5 M-sodium acetate, pH 3.7, and the mixture was incubated at 37°C for 120 h before it was dialysed against SSC, pH 7.0. The frequency of AP sites is indicated for each experiment.

Alkylation–depurination. [^{3}H]DNA in 0.5 M-sodium phosphate, pH 7.0, was alkylated with 0.3 M-methyl methanesulphonate at 37°C for 60 min. After dialysis against SSC, pH 7.0, it was heated at 50°C for 6 h and again dialysed. The alkylated-depurinated DNA contains about 60 AP sites/10^3 nucleotides (Paquette *et al.*, 1972).

5′-Phosphate labelling. To produce strand breaks limited by 5′-phosphate, DNA, labelled or not, was treated with pancreatic deoxyribonuclease in some experiments; in other experiments, depurinated DNA was treated with chromatin AP endonuclease.

The nicked DNA in 20 mM-NaCl/10 mM-Tris/HCl, pH 8.0, was incubated for 30 min at 37°C with 0.1 unit of alkaline phosphatase/μg of DNA. After two phenol deproteinizations, the DNA was incubated in 10 mM-$MgCl_2$/20 mM-β-mercaptoethanol/30 mM-Tris/HCl, pH 8.0, with 0.5 unit of polynucleotide 5′-hydroxykinase and 0.5 μCi of [γ-^{32}P]ATP/μg of DNA for 30 min at 37°C. After two phenol deproteinizations, the DNA was separated from the excess [γ-^{32}P]ATP either by ethanol precipitation or by Sephadex G-50 chromatography.

Poly(dA-[methyl-^{3}H]dT) labelled with ^{14}C at the 3′ ends. The ^{3}H-labelled double-stranded polynucleotide (9.5 μg/ml) was incubated in 6.2 mM-$MgCl_2$ / 0.9 mM-dithiothreitol / 125 mM-potassium phosphate, pH 7.4, with 1.2 units of Klenow fragment/μg of polynucleotide, 0.1 mM-dATP and, per ml, 0.22 μCi of [^{14}C]dTTP (452 mCi/mmol) for 120 min at 13°C. After two phenol deproteinizations, the doubly-labelled poly(dA-dT) was dialysed against 2 mM-$MgCl_2$/50 mM-Tris/HCl, pH 7.5; its specific radioactivities are 55700 d.p.m. of ^{3}H and 70000 d.p.m. of ^{14}C/μg.

Deoxyribonuclease IV assay

To 10 μl of poly(dA-[^{3}H]dT) (0.1 μg) solution were added 100 μl of 3 mM-$MgCl_2$/1 mM-dithiothreitol/0.05% bovine serum albumin/50 mM-Hepes/KOH, pH 7.5, and 10 μl of enzyme preparation. After 15 min at 37°C, the radioactivity soluble in 5% $HClO_4$ was measured. One enzyme unit releases 1 nmol of nucleotides in the acid-soluble fraction/15 min at 37°C (Lindahl *et al.*, 1969).

Chromatographies

Oligonucleotide separation. Separation of oligonucleotides according to length was performed as described by Junowicz & Spencer (1970). The chromatography was carried out at 22°C on a 70 cm × 0.9 cm column of DEAE-Sephadex A-25 equilibrated with 7 M-urea/5 mM-Tris/HCl, pH 7.6 (urea/Tris buffer). Absorbance markers (5′-mono-, di- and trinucleotides) were added to the sample containing the mixture of radioactive molecules to be analysed. The elution was performed with a linear 0–0.4 M-LiCl gradient in the urea/Tris buffer at a rate of 30 ml/h; A_{260} and radioactivity were measured on the collected 4 ml fractions.

Separation of deoxyribose 5-phosphate and mononucleotides. A 30 cm × 0.9 cm column of DEAE-Sephadex A-25 equilibrated with 0.1 M-Tris/HCl, pH 8.3, was used. The elution was carried out with a linear 0–0.2 M-NaCl gradient in the same buffer and 2 ml fractions were collected. 5′-Mononucleotides and deoxyribose 5-phosphate were added as markers to the sample containing the mixture of radioactive molecules to be analysed; the nucleotides were located by their A_{260}, whereas the deoxyribose 5-phosphate was located by reaction with diphenylamine (Schneider, 1957).

Chromatography on Bio-Gel P-100. A 1 cm × 120 cm column of Bio-Gel P-100 was equilibrated with 0.1 M-$(NH_4)_2SO_4$/0.1 mM-dithiothreitol/50 mM-Tris/HCl, pH 8.0. The dead volume (V_o) was measured with ferritin, the total volume (V_t) with riboflavin, and the column was calibrated with four proteins of known M_r (cytochrome *c*, 12500; chymotrypsinogen, 25000; ovalbumin, 45000; bovine serum albumin, 67000). Each protein was dissolved in 1 ml of buffer and placed on top of the column; the elution was carried out at a rate of 4 ml/h and the A_{230} was measured on each 1.1 ml fraction to determine the elution volume (V_e). Plotting $K_{av.} = (V_e - V_o)/(V_t - V_o)$ as a function of the logarithm of the M_r gave a straight line.

The enzyme preparation was dialysed against the same buffer and 1 ml of solution was chromatographed as above; the activity of the fractions on poly(dA-[^{3}H]dT) was measured to determine V_e; $K_{av.}$ was calculated and the calibration graph was used to determine the M_r.

Miscellaneous

Determination of Norit-non-adsorbable radioactivity. To the 20 μl sample was added 400 μl of 0.7 M-trichloroacetic acid, 100 μl of 20 mM-$Na_4P_2O_7$/25 mM-KH_2PO_4/0.05% bovine serum albumin and 200 μl of 20% Norit in water. After 5 min shaking at 4°C, the suspension was centrifuged for 30 min at 12000 *g* and the radioactivity was measured on the supernatant.

Determination of the acid-soluble fraction (in 5% $HClO_4$ at 0°C). To *x* μl of reacted radioactive DNA or poly(dA-dT) solution were added 100 μl of

SSC, pH7.0, containing 200μg of calf thymus DNA, and 2(x+100) μl of 7.5% $HClO_4$. After 5min on ice, the tube was centrifuged for 10min at 12000g and the radioactivity was measured on the supernatant.

Determination of AP sites with [^{14}C]methoxyamine. Methoxyamine reacts specifically with AP sites, forming an addition complex without nicking the DNA strands; [^{14}C]methoxyamine can thus be used to count AP sites. We follow the method described by Talpaert-Borlé & Liuzzi (1983). At the end of the reaction, the mixture (30μl) was placed on a glass-fibre filter (Whatman GFC, 2.5cm diameter) which was immersed in 1M-HCl. After several washings with the acid and ethanol, the filter was dried at 50°C and the radioactivity was counted. A [^{14}C]DNA standard was used to determine the counting yield. Knowing the specific radioactivity of the [^{14}C]methoxyamine, the number of AP sites can be calculated.

Results

Lindahl *et al.* (1969) have found that the exonuclease activity of DNAase IV is much greater on poly(dA-dT) than on nicked DNA. This is why the cellular localization of the activity on the synthetic substrate was first investigated. The chromatin activity was then purified and identified as DNAase IV.

Cellular localization of the poly(dA-dT) degrading activity

Rat liver was collected and homogenized in 5% dextran/2.5% Ficoll/4mM $CaCl_2$/2.4mM-EDTA/0.25M sucrose/1mM-2-mercaptoethanol/0.1% bovine serum albumin, pH7.0, as described by Thibodeau & Verly (1980). After centrifugation, the crude nuclei contained 94% of the activity. These nuclei were purified and the chromatin separated from nucleosol also according to Thibodeau & Verly (1980); the chromatin was dissociated with heparin–Ultrogel and the non-histone proteins extracted with 10mM-potassium phosphate/0.5M-KCl/10mM-Tris/HCl, pH8.0, as described by Renard & Verly (1980). The nucleosol contained 58% of the nucleus activity and the chromatin non-histone proteins 42%.

Purification of the poly(dA-dT)-degrading activity of chromatin

The chromatin non-histone protein was chromatographed on phosphocellulose (Fig. 1*a*). Fractions 77–81, containing the activity on poly(dA-[^{3}H]dT), were pooled and chromatographed on hydroxyapatite (Fig. 1*b*). The active fractions 52–54 were pooled, dialysed against 1mM-EDTA / 0.2mM-dithiothreitol / 20mM-Tris / HCl, pH8.0, and, after addition of glycerol up to 40%, kept at −20°C. Aliquots were taken from the different solutions for protein determination (Peterson, 1977); Table 1 summarizes the three steps of the enzyme purification.

Some properties of the enzyme

These properties were studied using poly(dA-[^{3}H]dT) as substrate.

Buffers of 50mM-Tris / 50mM-Hepes / 4mM-$MgCl_2$/1mM-dithiothreitol were adjusted to different pH values from 6 to 9 by addition of 12M-HCl or 10M-KOH. To 100μl of buffer of a given pH were added 10μl of poly(dA-[^{3}H]dT) solution (0.1μg of polynucleotide) and 10μl of the purified enzyme preparation (0.09unit). After 15min at 37°C, the acid-soluble radioactivity was measured. The optimum pH of the degrading enzyme was found to be 7.5.

Buffers of 50mM-Hepes / KOH / 1mM-dithiothreitol, containing 0–20mM-$MgCl_2$, were adjusted to pH7.5. To 100μl of buffer of a given Mg^{2+} concentration were added substrate and enzyme as above. The optimal Mg^{2+} concentration was between 2 and 4mM.

The enzyme is inhibited by ethylmaleimide. Its M_r, determined on Bio-Gel P-100, was 58000.

The enzyme is a 5′–3′ exonuclease degrading double-stranded DNA

Several tubes were prepared containing, in 100μl of 2mM-$MgCl_2$/1mM-dithiothreitol/0.05% bovine serum albumin/50mM-Hepes/KOH, pH7.5 (buffer D), 0.09 unit of the purified enzyme and 0.1μg of one of the following substrates: untreated [^{3}H]DNA, [^{3}H]DNA treated with pancreatic deoxyribonuclease, poly(dA-[^{3}H]dT). The acid-soluble radioactivity was measured after various times at 37°C and, after correction for the zero-time value, expressed as a percentage of the total radioactivity.

Fig. 2 shows that nicked DNA is a much better substrate than intact DNA, which suggests the presence of an exonucleolytic activity. On the other hand, the [^{3}H]DNA treated with pancreatic deoxyribonuclease and the poly(dA-[^{3}H]dT), before incubation with the chromatin enzyme, gave about the same amounts of acid-soluble radioactivity (2.8 and 2.5% respectively) which means that they had approximately the same average break frequencies (about 15 breaks/10^3 nucleotides). Fig. 2 however shows that the synthetic polynucleotide was degraded much faster than the DNA, in agreement with the observation of Lindahl *et al.* (1969) on DNAase IV of rabbit bone marrow.

Samples of [^{3}H]DNA treated with pancreatic deoxyribonuclease (17 breaks/10^3 nucleotides) and

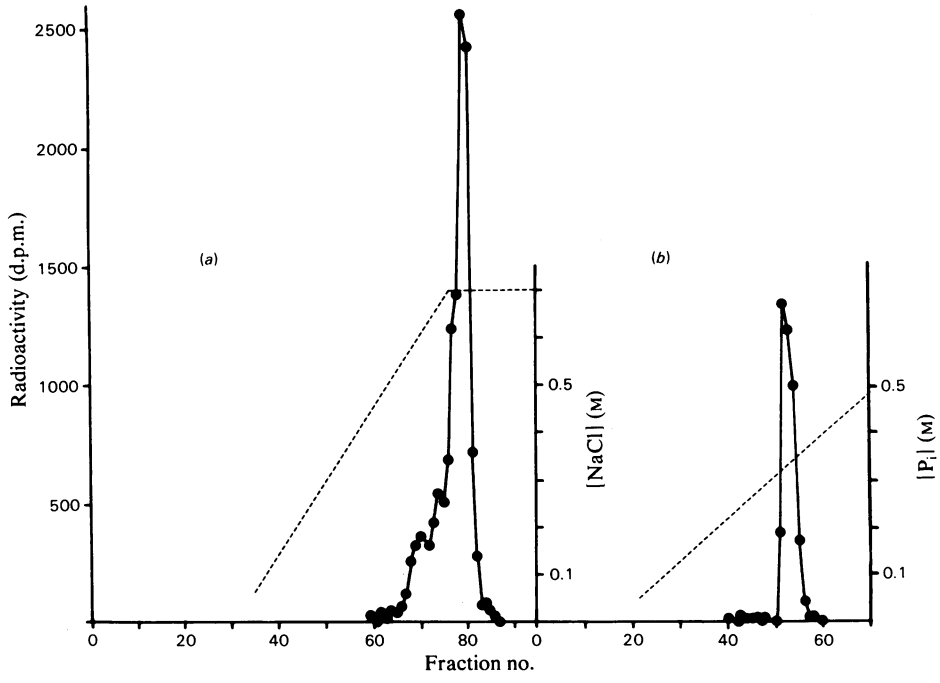

Fig. 1. *Purification of the poly(dA-dT)-degrading activity of chromatin*

(*a*) The chromatin non-histone protein (2.65mg) solution (26ml), prepared from 8g of rat liver, was dialysed against 0.5mM-EDTA/1mM-dithiothreitol/10mM-sodium phosphate, pH7.2 (buffer A), then placed, at a rate of 10ml/h, onto a 1.6cm × 30cm phosphocellulose column. After washing with 15ml of buffer A and 60ml of buffer B (0.5mM-EDTA/1mM-dithiothreitol/20mM-sodium phosphate, pH7.2) containing 0.04M-NaCl, the column was eluted with 150ml of a linear 0.04–0.70M-NaCl gradient, then with 60ml of 0.70M-NaCl in buffer B, and 2.8ml fractions were collected. (*b*) Fractions 77–81 from the previous chromatography (14ml; 530μg of protein) were pooled and layered on top of a 0.9cm × 6cm hydroxyapatite column equilibrated with 50mM-potassium phosphate, pH7.5 (buffer C) at a rate of 10ml/h; the column was carefully washed with buffer C, then eluted with 100ml of a linear 0.05–0.50M-potassium phosphate gradient, pH7.5, always at the same rate. Fractions (1.6ml) were collected. In both cases, the activity on poly(dA-[^{3}H]dT) was measured and recorded as acid-soluble d.p.m. of ^{3}H per 10μl aliquot from each fraction (●). ---, Gradient.

Table 1. *Purification of the chromatin activity degrading poly(dA-dT)*

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)
Chromatin non-histone proteins	2.65	136	51
After phosphocellulose	0.53	51	96
After hydroxyapatite	0.07	19	274

labelled with ^{32}P at the 5′-phosphate ends (2.5μg) and 0.25 unit of the enzyme preparation in 100μl of buffer D were incubated at 37°C for various times before measuring the acid-soluble radioactivity. Controls without enzyme were carried out and used to correct the data. Table 2 shows that ^{32}P was released from the doubly-labelled DNA much faster than ^{3}H. An analysis, on DEAE-Sephadex A-25 in the presence of 7M-urea, of the acid-soluble radioactivity after 30min of incubation showed that the ^{32}P was mainly in mononucleotides. The conclusion is that the enzyme is an exonuclease degrading double-stranded DNA from nicks in the 5′–3′ direction. From now on, we shall call the enzyme 'chromatin DNAase IV'.

Samples of poly(dA-dT), labelled with ^{3}H except for the 3′ ends, which were labelled with ^{14}C (see the Material and methods section) (0.7μg) and 0.09 unit of enzyme preparation in 100μl of buffer D were incubated at 37°C for various times before the acid soluble radioactivity was measured. Controls without enzyme were carried out and used to correct the data. Table 2 shows that ^{3}H was released from the doubly-labelled polymer faster than ^{14}C, which is in agreement with a 5′–3′ exonucleolytic activity. However, when the acid-soluble radioactivity obtained after a 15min incubation was analysed on DEAE-Sephadex A-25

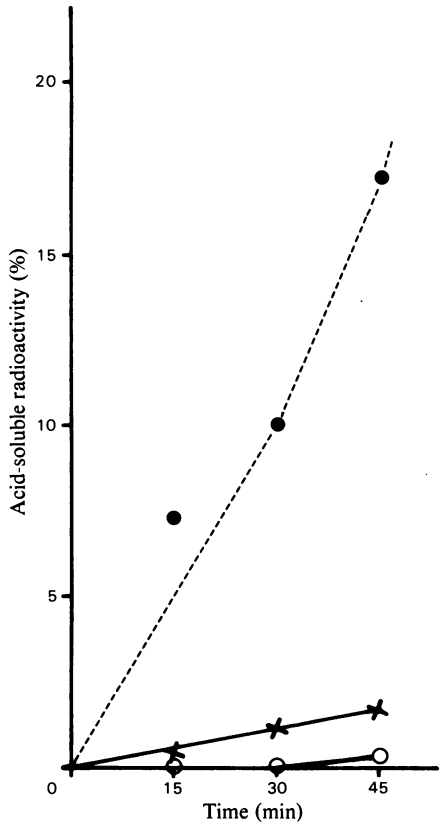

Fig. 2. *Exonucleolytic activity of the purified chromatin enzyme*

The enzyme (0.09 unit) was incubated with 0.1 μg of untreated [^{3}H]DNA (○), [^{3}H]DNA nicked with pancreatic DNAase (×), or poly(dA-[^{3}H]dT) (●). The acid-soluble radioactivity, corrected for the zero-time value, is plotted as a percentage of total radioactivity versus incubation time (min).

Table 2. *Hydrolysis of two doubly-labelled substrates by the chromatin enzyme*

Time (min)	Total radioactivity in acid-soluble fraction (%)			
	[^{3}H]DNA labelled with ^{32}P on the 5′-phosphate		Poly(dA-[^{3}H]dT) with ^{14}C-labelled 3′ ends	
	^{32}P	^{3}H	^{3}H	^{14}C
0	0.0	0.00	0.0	0.0
5	1.9	0.00	1.4	0.2
10	3.2	0.10	5.5	1.2
15	4.5	0.14	6.4	1.6
30	9.2	0.18	17.5	4.7
60	11.0	0.80	—	—
120	15.2	1.20	—	—

in the presence of 7M-urea, part of the ^{14}C was found in mono- and di-nucleotides, whereas radioactive tri- and tetra-nucleotides were absent. The preparation thus has a limited 3′–5′ exonuclease activity, but there is no doubt that the 5′–3′ exonuclease is dominant. We do not know whether this 3′–5′ exonuclease activity is a contamination in the preparation or an intrinsic property of the chromatin DNAase IV. We have, in particular, considered the possibility that the chromatin DNAase IV preparation might contain DNAase V, an exonuclease working in both directions 5′–3′ and 3′–5′ (Mosbaugh & Meyer, 1980); DNAase V also easily degrades poly(dA–dT). It seems unlikely that DNAase V was a contaminant in our preparation since it has an M_r of 12000 and the chromatography of our preparation on Bio-Gel P-100 showed a single activity peak on poly(dA-dT) corresponding to an M_r of 58000. We must also emphasize that the non-histone proteins of rat liver chromatin seem to contain little DNAase V since there was a single peak of activity on poly(dA-dT) in both chromatographies used for the purification of DNAase IV.

AP site excision

Alkylated–depurinated [^{3}H]DNA (60 AP sites/10^3 nucleotides) was treated or not with the chromatin AP endonuclease. The DNA samples were then dialysed against buffer D. Aliquots (19 μg of DNA) were exposed to 0.2 unit of chromatin DNAase IV in 100 μl of incubation medium. After incubations from 0 to 90 min at 37°C, the AP sites were determined with [^{14}C]methoxyamine. Table 3 gives the ratio ^{14}C/^{3}H in the 1M-HCl-insoluble fraction. This ratio did not change with time when the alkylated–depurinated [^{3}H]DNA had not been treated with the AP endonuclease; however, when the DNA strands had been incised 5′ to the AP sites by the AP endonuclease, an incubation with the chromatin DNAase IV led to a decrease of the ratio. This means that, after the 5′ incision by the AP endonuclease, DNAase IV was able to excise the AP sites.

The AP site excision product

Acid-depurinated DNA (10 AP sites/10^3 nucleotides) was treated with the chromatin AP endonuclease to place a break 5′ to the AP sites, then reduced with NaB^3H_4. Samples of this substrate (0.4 μg) and the chromatin DNAase IV (0.1 unit) in 100 μl of buffer D were incubated at 37°C for periods of time up to 24 h. No increase of acid-soluble ^{3}H radioactivity was observed. The conclusion was that the reduced AP site was not excised.

Alkylated–depurinated [^{3}H]DNA (60 AP sites/

Table 3. *Excision of AP sites by chromatin DNAase IV from alkylated–depurinated DNA nicked with the chromatin AP endonuclease*

The alkylated–depurinated [^{3}H]DNA was treated or not with the AP endonuclease, incubated with chromatin DNAse IV for various periods of time, then reacted with [^{14}C]methoxyamine. The ^{14}C and ^{3}H radioactivities are measured on the 1M-HCl-insoluble fraction. The lower ^{14}C/^{3}H ratio found in the zero-time incubation with DNAase IV when the substrate had been treated with the AP endonuclease is explained by a loss of acid-soluble fragments with an AP site frequency higher than average since each fragment, short or long, has an AP site at its 5′ end.

AP endonuclease treatment	Incubation with DNAase IV (min)	^{14}C (c.p.m.)	^{3}H (c.p.m.)	^{14}C/^{3}H
–	0	3014	13177	0.229
	15	2865	12031	0.238
	30	3058	13175	0.232
	60	3214	13448	0.239
	90	3122	13148	0.237
+	0	854	5284	0.162
	15	710	4775	0.144
	30	617	4363	0.141
	60	488	3517	0.139
	90	458	3357	0.136

10^3 nucleotides) was exposed to the chromatin AP endonuclease, then the 5′-phosphate ends were labelled with ^{32}P. Incubation of this substrate (15μg) with chromatin DNAase IV (1.4units) in 500μl of buffer D increased the acid-soluble ^{32}P. Analysis of the acid-soluble fraction after a 15min incubation at 37°C on DEAE-Sephadex A-25 in the presence of 7M-urea showed that most of the ^{32}P was eluted with the mononucleotides (results not shown). This suggested that the AP site might be excised as deoxyribose 5-phosphate by hydrolysis of the phosphodiester bond on its 3′ side and not as deoxyribose 5-phosphate covalently bound to one or two nucleotides, as is the case when the AP site is excised by the 5′–3′ exonuclease activity of *E. coli* DNA polymerase I (Gossard & Verly, 1978). The incubation mixture was also chromatographed on DEAE-Sephadex A-25 in the absence of urea, in conditions that allowed to separate deoxyribose 5-phosphate from the mononucleotides. Fig. 3(*a*) shows that part of the ^{32}P was eluted with the mononucleotides; however, another ^{32}P peak (fraction 29) was eluted one fraction after the peak of the deoxyribose 5-phosphate marker (fraction 28). The interpretation is that the radioactive mononucleotides are the result of strand degradation from parasitic breaks, whereas degradation from the nicks placed 5′ to

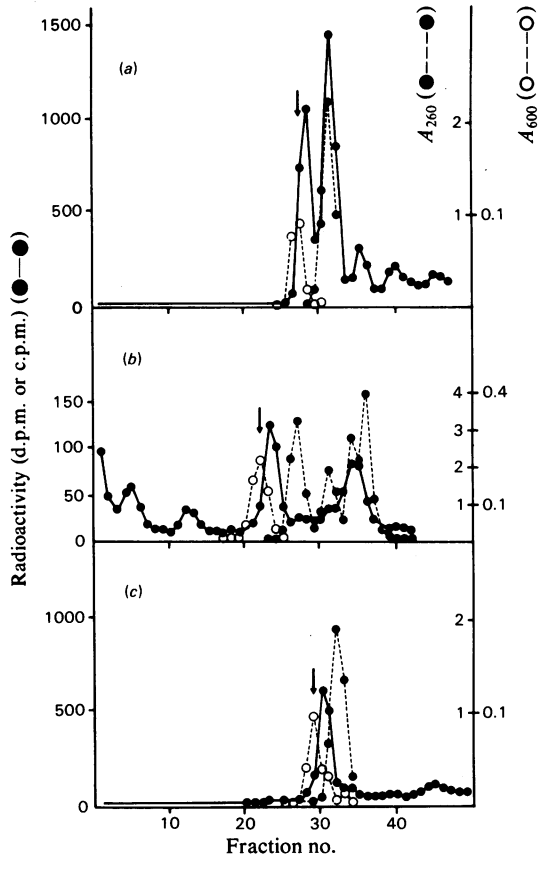

Fig. 3. *The AP site excision product given by chromatin DNAase IV*

(*a*) Alkylated–depurinated DNA treated with chromatin AP endonuclease, then labelled with ^{32}P on the 5′-phosphate ends, was incubated with chromatin DNAase IV. (*b*) Acid-depurinated DNA labelled with ^{3}H in the 2, 8 and 5′ positions of deoxyadenosine residues, treated with chromatin AP endonuclease, was incubated with chromatin DNAase IV. (*c*) Alkylated–depurinated DNA treated with chromatin AP endonuclease, then labelled with ^{32}P on the 5′-phosphate ends, was incubated at pH11. The three incubation mixtures, after addition of deoxyribose 5-phosphate and 5′-monodeoxynucleotides as markers, were chromatographed on DEAE-Sephadex A-25 in absence of urea. (●—●), Elution pattern of the radioactivity: ^{32}P in (*a*) and (*c*) (d.p.m./0.5ml from each fraction) and ^{3}H in B (c.p.m./2ml from each fraction). (●---●), A_{260}: dTMP, dGMP and dAMP appear in that order in (*a*); dTMP, dGMP, dAMP and dCMP in (*b*); there is only a peak of dTMP in (*c*). (○---○), A_{600} after reaction with diphenylamine; the elution position of the deoxyribose 5-phosphate marker is indicated by an arrow.

the AP sites by the AP endonuclease does not yield deoxyribose 5-phosphate but a related molecule.

In another experiment, DNA labelled with ^{3}H

on the 2, 8 and 5′ positions of the deoxyadenosine residues was used. After acid-depurination (17 AP sites/10^3 nucleotides), it was exposed to the chromatin AP endonuclease. This substrate (15 μg) and the chromatin DNAase IV (2 units) in 500 μl of buffer D were incubated at 37°C for 15 min before analysing the reaction mixture on DEAE-Sephadex A-25 in absence of urea. Fig. 3(*b*) shows a ^{3}H peak in the position of dAMP (fraction 34) and another one (fraction 23) which is one fraction after the peak of the deoxyribose 5-phosphate marker (fraction 22), confirming that the AP site is excised by DNAase IV not as deoxyribose 5-phosphate, but in a related molecule.

Alkylated–depurinated [^{3}H]DNA (60 AP sites/10^3 nucleotides), nicked with the AP endonuclease and labelled with ^{32}P on the 5′-phosphate ends, was exposed to pH 11 for 90 min at 37°C. The alkaline treatment increased the acid-soluble ^{32}P, but not the acid-soluble ^{3}H. Chromatography of the reaction mixture on DEAE-Sephadex A-25 in absence of urea (Fig. 3*c*) gave a ^{32}P peak (fraction 30) which was one fraction after the peak of the deoxyribose 5-phosphate marker (fraction 29). It is known that, after nicking with an AP endonuclease, an alkaline treatment releases the AP site as a 2,3-unsaturated derivative of deoxyribose 5-phosphate (Grossman & Grafstrom, 1982).

The ^{32}P molecules emerging immediately after the deoxyribose 5-phosphate marker in the chromatographies on DEAE-Sephadex A-25 in absence of urea were further analysed. Whatever the releasing procedure [alkaline pH (Fig. 3*c*) or chromatin DNAase IV (Fig. 3*a*)], the ^{32}P radioactivity was not adsorbed on Norit; moreover, the released ^{32}P fragments behaved identically on cellulose t.l.c. eluted with methanol/conc. NH_4OH/water (6:1:3 by vol.) (results not shown).

Discussion

The activity degrading poly(dA-dT) in rat liver is mainly concentrated in cell nuclei where it is nearly equally distributed between nucleosol and the non-histone proteins of chromatin.

The activity in the non-histone proteins of chromatin was chromatographed successively on phosphocellulose and hydroxyapatite. There was, in each case, a single peak of activity which suggests that, in chromatin, there is only one main enzyme degrading poly(dA-dT).

This enzyme was found to be an exonuclease degrading double-stranded DNA from nicks in the 5′–3′ direction. The chromatin enzyme is thus analogous to DNAase IV purified by Lindahl *et al.* (1969) from rabbit bone marrow. Like the rabbit enzyme, it is more active on poly(dA-dT) than on nicked DNA; it has the same behaviour on phosphocellulose and hydroxyapatite; its M_r of 58000 can be compared with the 42000 of the rabbit enzyme. Our purified enzyme will thus be called chromatin DNAase IV of rat liver. The optimal pH is 7.5 and the optimal Mg^{2+} concentration between 2 and 4 mM; the chromatin DNAase IV is inhibited by ethylmaleimide. Our preparation of chromatin DNAase IV has a slight 3′–5′ exonuclease activity.

Goffin & Verly (1982, 1984) have repaired DNA containing AP sites by successive incubations with the AP endonuclease, DNAase IV, DNA polymerase β prepared from rat liver chromatin, and phage T4 DNA ligase. This result suggests that, after incision on the 5′ side of the AP site by the AP endonuclease, chromatin DNAase IV was capable of excising the AP site. We have indeed shown that the AP sites disappear from the incised DNA during the incubation with the chromatin DNAase IV by determining the remaining AP sites with [^{14}C]methoxyamine.

We next wanted to know in what molecule the AP site was released from the nicked DNA by the chromatin DNAase IV. The results obtained with DNA labelled with ^{32}P on the 5′-phosphate ends produced by the AP endonuclease or with ^{3}H in the base-free deoxyribose indicated that DNAase IV excised the AP site not as deoxyribose 5-phosphate but in a related molecule (Figs. 3*a* and 3*b*).

Since reduction of the AP site with $NaBH_4$ in a DNA incised with the AP endonuclease prevented its excision by chromatin DNAase IV, we wondered whether the enzyme would not catalyse a β-elimination, releasing the AP site as the 2,3-unsaturated derivative of deoxyribose 5-phosphate. Alkaline pH is known to induce β-elimination. We thus could, after labelling with ^{32}P the 5′-phosphate ends created by the AP endonuclease, prepare a ^{32}P-labelled standard of the 2,3-unsaturated derivative of deoxyribose 5-phosphate by a treatment with NaOH (Grossman & Grafstrom, 1982). This standard behaved exactly as the molecule released by the chromatin DNAase IV on DEAE-Sephadex A-25 in absence of urea (Fig. 3*c*) and on cellulose t.l.c.

The final conclusion is that, to excise the AP site from DNA nicked with the AP endonuclease, the chromatin DNAase IV does not act as a nuclease that hydrolyses the phosphodiester bond 3′ of the AP site to yield 3′-hydroxy and 5′-phosphate ends, but as a catalyst of β-elimination; no 3′-hydroxy end is created but rather a 2′–3′ double bond. It is important to notice that, if the excision of AP sites from nuclear DNA depends on β-elimination, DNAase IV is not the only catalyst available in chromatin; molecules like histones and polyamines might play a more important role.

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