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Studies on Rat Liver Nuclear DNA Damaged by Chemical Carcinogen (3'-Me DAB) and AP DNA Endonuclease. I. Purification and Some Properties of AP DNA Endonucleases in Rat Liver Chromatin.*

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Three kinds of apurinic/apyrimidinic (AP) DNA endonuclease, APcI, APcII, APcIII, were purified from rat liver chromatin through 1M KCl extraction, DEAE-trisacryl ion exchange chromatography, Sephadex G-150 gel filtration and AP DNA cellulose affinity chromatography. Activities of the purified APcI, APcII and APcIII were 62.5, 83.3 and 52.0 EU/mg of protein, respectively.

Molecular weights of APcI, APcII and APcIII, each consisting of a single polypeptide, were 30,000, 42,000 and 13,000, and isoelectric points of them were 7.2, 6.3 and 6.2, respectively. Three enzymes showed different substrate specificities; APcI acted only on AP DNA, and APcII acted on both AP DNA and UV DNA, while APcIII acted on 3'-methyl-4-monomethylaminoazobenzene (3'-Me MAB) DNA adduct as well as AP DNA and UV DNA.

These results indicate that three kinds of AP DNA endonuclease present in rat liver chromatin have structural and functional diversities.

Key Words: *AP DNA endonuclease, Purification, Rat liver chromatin.*

INTRODUCTION

Apurinic or apyrimidinic (AP) lesions in DNA are made from spontaneous depurination (Lindahl and Nyberg, 1972) or from the action of glycosylases acting on DNA with various modified bases (Lindahl, 1979). The excision of pyrimidine dimer by *M.luteus* or T_4 UV endonucleases may produce an apyrimidinic site that is recognized by AP DNA endonucleases (Grossman et al., 1978, Demple and Linn, 1980, Haseltine et al., 1980).

The damage in DNA by physical or chemical agents must be repaired by DNA repair system in normal cells or mutation may result from the loss of the fidelity in genetic information during unfaithful DNA replication (Sharman and Loeb, 1979). AP DNA endonucleases (EC 3.1.25.2), recognizing AP sites and nicking the DNA strands near AP sites, have been shown to be present in all cells, prokaryotic and eukaryotic (Brent, 1976).

It has been reported that most of the rat liver AP DNA endonuclease activity is located in chromatin (Thibodeau and Verly, 1980), and that two species of the enzyme are present in the non-histone protein fraction in rat liver chromatin (Thibodeau et al., 1980). Two classes of AP DNA endonucleases which have different cleavage specificities have also been isolated from cultured human fibroblasts (Mosbaugh and Linn, 1980).

In our preliminary work (Lee et al., 1986), we demonstrated that more than two species of AP DNA endonucleases were present in rat liver chromatin, but their nature was not characterized.

This paper presents a study on the purification of

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3 kinds of AP DNA endonuclease from rat liver chromatin and their substrate specificities for DNA damaged by 3'-methyl-4-monomethylaminoazobenzene (3'-Me MAB), an immediate metabolite of 3'-methyl-4-dimethylaminoazobenzene (3'-Me DAB) that is a hepatocarcinogene, UV or heat under the acidic condition.

MATERIALS AND METHODS

Materials

Sprague Dowley male rats (-150g body weight) fed with a commercial rat diet (Je Ill Je Dang, Korea) were used for the enzyme preparation. [^{3}H]-thymidine (specific activity; 2Ci/mmole) was purchased from Amersham Co., England and phenylmethylsulfonyl fluoride (PMSF), agarose, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), cellulose, ethylene diaminetetracetic acid (EDTA), tris(hydroxymethyl) aminomethane (Tris) and dialysis bag were purchased from Sigma Chemical Co., U.S.A. Sephadex G-150 and DEAE Trisacryl were products of Pharmacia Fine Chem., Sweden.

3'-Methyl-4-monomethylaminoazobenzene (3'-Me MAB) was a gift from Dr. J. Miller (University of Wisconsin).

Preparation of substrate

AP DNA: *Eschericia coli* (HB101) was cultured in LB media containing [^{3}H]-thymidine (30μCi/100ml) for 24 hours and harvested. ^{3}H labeled *E.coli* chromosomal DNA was isolated by the method of Dale and Greenaway (1984). [^{3}H] AP DNA was prepared by the treatment of DNA dissolved in 1.0M acetate buffer, pH 4.0, with heat (70°C) for 2 hours, followed by the precipitation with cold ethanol (75%).

The number of AP sites in DNA was calculated from the equation of Zubroff and Sarma (1976):

No. AP site/molecule=Mn (control DNA)/Mn (excised AP DNA)−1; where Mn (number average molacular weight)= Σ Wi/Σ Wi/Mi,

Wi: weight of DNA in each fraction,
Mi: molecular weight of DNA in each fraction

Mn of control DNA was determined after alkaline agarose gel electrophoresis (0.8%), and Mn of excised AP DNA was determined by polyacrylamide urea gel electrophoresis of excised AP DNA by alkaline treatment (pH 12.3). Enzyme unit was defined as pmoles of substrate (AP site) excised per min.

The radioactivity in the supernatant after alkali treatment of [^{3}H] AP DNA was assumed to be 100% AP site excised, and the relative radioactivity in the supernatant after the enzyme reaction was used for the calculation of enzyme unit.

3'-Me MAB adduct:

3'-Me MAB DNA adduct was synthesized in vitro according to the method of Lin and Fok (1973). To 3ml of 0.05M sodium phosphate buffer (pH 7.0) containing 33% ethanol, 0.42mM 3'-Me MAB and 4.0mM potassium persulfate, 0.15mg of [^{3}H] *E.coli* chromosomal DNA (10,000dpm/10μg) was added and reacted for 14 hours at 25°C.

3'-Me MAB DNA adduct was precipitated with 2 volumes of ethenol containing 0.05M NaCl and washed twice with the same volume of ether and 70% of ethanol.

The formation of 3'-Me MAB DNA adduct was confirmed by the high pressure liquid chromatography (HPLC; LDC, U.S.A)

[^{3}H] UV DNA:

[^{3}H] UV DNA was prepared by an exposure of UV light (600J/m^2) onto the [^{3}H] *E.coli* DNA (1.4mg/10ml of 10mM Tris-HCl buffer, pH 8.0) in plastic petri dish (10cm ID) on the ice.

The formation of pyrimidine dimer was confirmed by HPLC.

Enzyme assay

AP DNA endonuclease activity was measured by the method of Thibodeau et al. (1980). To the [^{3}H] AP DNA dissolved in 100μl of Tris-HCl buffer, pH8.0, 100μl of enzyme solution was added and reacted for 15min at 37°C. The reaction was stopped by transfering the reaction mixture to ice-cold water, and then 100μl of 200mg% of bovine serum albumin solution and 60μl of 30% perchloic acid were added to the reaction mixture, and then mixed.

After standing for 15min at 0°C, the mixture was centrifuged for 15min at 10,000x*g* and radioactivities in the supernatant were counted in a liquid scintillation counter (Tricarb 300 Packard).

Purification of AP DNA endonucleases from rat liver chromatin

Preparation of liver chromatin: Liver was homogenized with 2 volume of 0.05M Tris potassium-magnesium buffer (0.05M Tris-HCl, pH7.5, 0.02M KCl, 0.005M $MgCl_2$, TKM) containing 0.25M sucrose and the homogenate was centrifuged at 800x*g* for 10min to precipitate the crude nuclear fraction.

Crude nuclear fraction dissolved in TKM buffer

(7ml/g liver) containing 2.2M sucrose was centrifuged at 53,000x*g* for 15min to sediment the liver nuclei according to the method of Blobel and Potter (1966).

Liver chromatin was obtained by the disruption of the nuclei by the addition of 0.5mM PMSF solution (1ml/g liver), followed by the centrifugation at 40,000×*g* of 30min to sediment the chromatin.

KCl extraction: To the chromatin fraction, 1M KCl solution containing 0.5mM PMSF and 5mM Tris-HCl buffer (pH8.0) was added to a final concentration of 5ml/g liver, and then mixed with stirring for 30min at 0°C. The mixture was centrifuged at 40,000x*g* for 30min and the clear supernatant was dialyzed at 4°C for 24 hrs against an excess amount of 50mM Tris-HCl buffer (pH7.0) containing 0.1mM EDTA and 0.1mM 2-mercaptoethanol (TEM). The dialysate was centrifuged at 10,000x*g* for 15min to remove the denatured protein precipitate and the supernatant was used as the enzyme source.

DEAE Trisacryl column chromatography: The chromatin extract was applied to a DEAE Tris-acryl column (2.6X25cm) equilibrated with TEM buffer at a flow rate of 20ml/hr and the column was washed with 100ml of the same buffer.

Then the column was eluted with a linear gradient of 0-0.4M NaCl in TEM buffer at a flow rate of 20ml/hr. Fractions containing AP DNA endonuclease activity were pooled and concentrated to a small volume by Amicon ultrafiltration (PM 10 membrane) system.

Gel filtration: The concentrated enzyme solution was applied to a Sephadex G-150 column (2.6X102cm) equilibrated with 10mM TEM buffer and eluted at a flow rate of 8ml/hr with the same buffer. Fractions containing the enzyme activity were pooled and saved for the next step.

AP DNA cellulose affinity chromatography: AP DNA cellulose was made by the method of Alberts and Herrick (1971). To 9mg of *E.coli* chromosomal AP DNA dissolved in 3ml of 10mM Tris-HCl buffer (pH 7.4) containing 1mM EDTA (TE buffer), 1g of cellulose was added, mixed and dried on a porcelain dish at 0°C. The dried cellulose powder was dissolved in 20ml of TE buffer, equilibrated for 24 hrs at 4°C and washed twice with TE buffer. The gel was packed into a column (0.5X5cm) and equilibrated with 20mM Tris-HCl buffer (pH 8.1) containing 0.1mM EDTA, 0.4mM 2-ME and 10% glycerol.

The pooled sample of AP DNA endonuclease were applied on the column and eluted with a stepwise gradient of 0, 0.2, 0.3 and 0.5M KCl in TE buffer at a flow rate of 4ml/hr.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). One hundred μl of each samples (50μg of protein, each) were mixed with an equal volume of a sample buffer (2% SDS, 5% 2-ME 10% glycerol), boiled for 2min in water bath, and then cooled.

Electrophoresis was run at a constant 30mA current. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue in 50% methanol and 10% acetic acid, followed by the destaining with 30% methanol and 10% acetic acid solution.

Isoelectric Focusing

Isoelectric points of the purified AP DNA endonucleases were determined by the electrophoresis on Ampholine Pag plate (pH 3.5-9.5, LKB, Sweden) according to the method of Fawett and Chrambach (1986). Five μg of the enzyme protein was separated on the plate at 1,000 volts of electricity for 2 hours and the position of the migrated protein band along the pH gradient was visulalized by staining and destaining as described in SDS-PAGE.

Determination of protein and DNA

Protein content in the enzyme preparation was determined by the method of Lowry et al. (1951), and DNA was quantitated by the method of Schneider (1957).

RESULTS

Preparation of Substrates

[^{3}H] AP DNA prepared from *E.coli* chromosomal DNA had a specific activity of 3.226 dpm/μg AP DNA and the number average molecular weight was estimated to the 4,079.8 kilodaltons (kDa) or 12,357 bases (Fig. 1).

The number average molecular weight of AP DNA excised by alkali treatment (pH 12.3) was estimated to be 69.1 kDa and the number of AP sites per molecule was calculated as 58 (Fig. 2). N-(guan-8-yl) 3'-Me MAB in DNA was the main 3'-Me MAB-modified base component of the hydrolysate of 3'-Me MAB DNA adduct, indicating that 3'-Me MAB bound mainly to No. 8 position of guanine ring in DNA.

APcI acted only on AP DNA, while APcII and APcIII acted on UV DNA as well as AP DNA. APcIII acted not only on AP DNA and UV DNA but also on 3'-Me MAB DNA adduct (Fig. 3).

Formation of thymine dimer in [^{3}H] UV DNA was observed and the relative amount of thymine dimer was 5% of total thymine present in *E.coli* chromosomal DNA (Fig. 4).

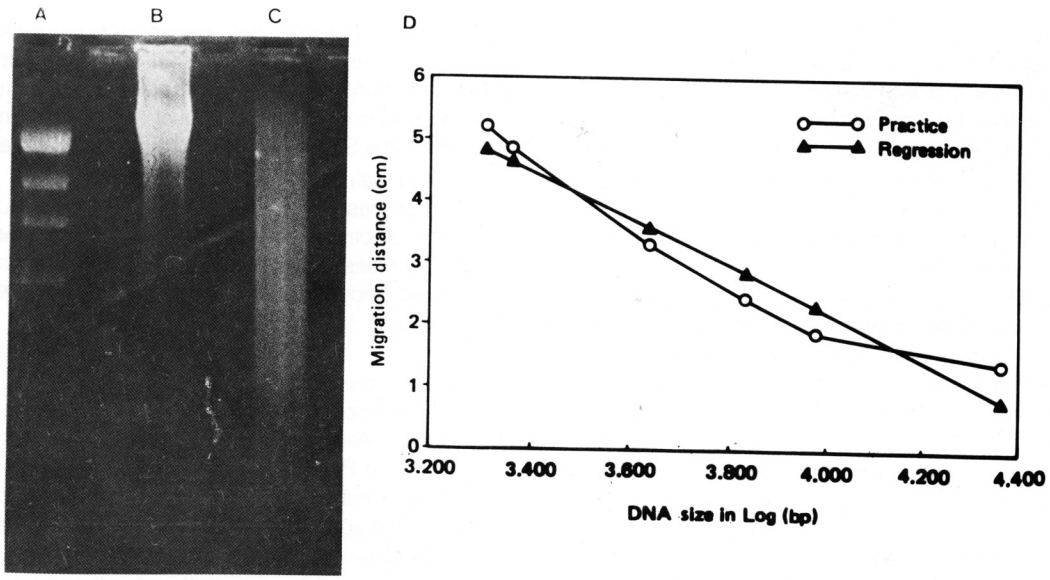

Fig. 1. Alkaline agarose gel (0.8%) electrophoresis of *E. coli* chromosomal DNA.
A, HindIII digested λ DNA molecular weight size marker (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kbps from above).
B, *E. coli* chromosomal DNA (20 μg).
C, *E. coli* chromosomal AP DNA (20 μg).
D, Standard curve for DNA molecular weight determination.

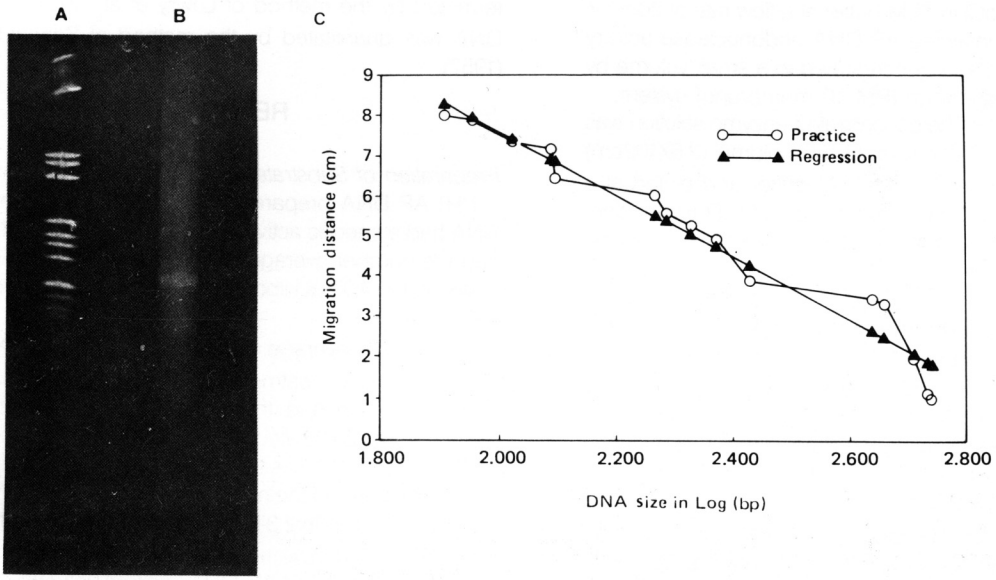

Fig. 2. Polyacrylamide urea gel (6%) electrophoresis of AP DNA excised by alkaline treatment.
A, Hae III digested pBR322 DNA molecular weight size marker (587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57 base pairs from above).
B, *E. coli* chromosomal AP DNA excised by alkaline treatment (pH 12.3).
C, Standard curve for DNA molecular weight determination.

$$\text{No. of AP site} = \frac{\text{Mn (control)}}{\text{Mn (excised AP DNA)}} - 1 = 58/\text{molecule}$$

$$Mn = \frac{\Sigma Wi}{\Sigma\left(\frac{Wi}{Mi}\right)} = 69.1 \text{ kDa}$$

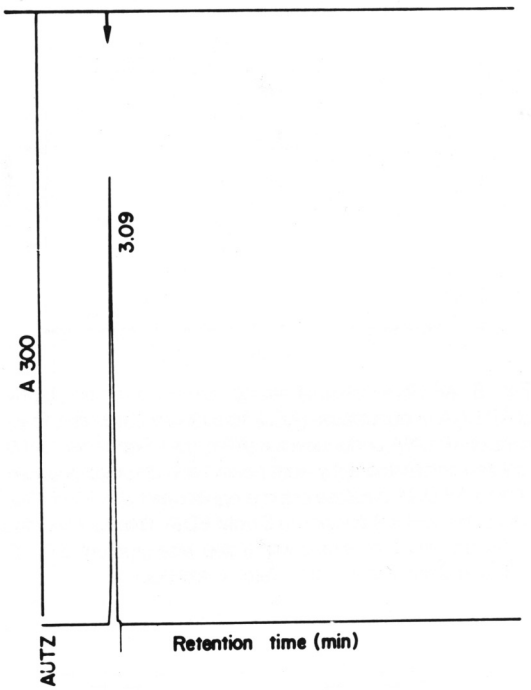

Fig. 3. Endonucleolytic activity of APcI, APcII and APcIII on AP DNA, 3'-Me-MAB DNA adduct and UV DNA. Every substrates (2μg DNA) were incubated with each enzyme (1 EU) for 30 min at 37°C and then the reaction products were electrophoresed on 0.8% agarose gel. Lane 1, DNA size marker (λ phage-HindIII digest); lane 2, AP DNA without any enzyme; lane 3, normal DNA+APcI; lane 4, AP DNA+APcI; lane 5, 3'-Me-MAB DNA+APcI; lane 6, UV DNA+APcI; lane 7, normal DNA+APcII; lane 8, AP DNA+APcII; lane 9, 3'-Me-MAB DNA+APcII;, lane 10, UV DNA+APcII; lane 11, normal DNA+APcIII; lane 12, AP DNA+APcIII; lane 13, 3'-Me-MAB DNA+APcIII; lane 14, UV DNA+APcIII.

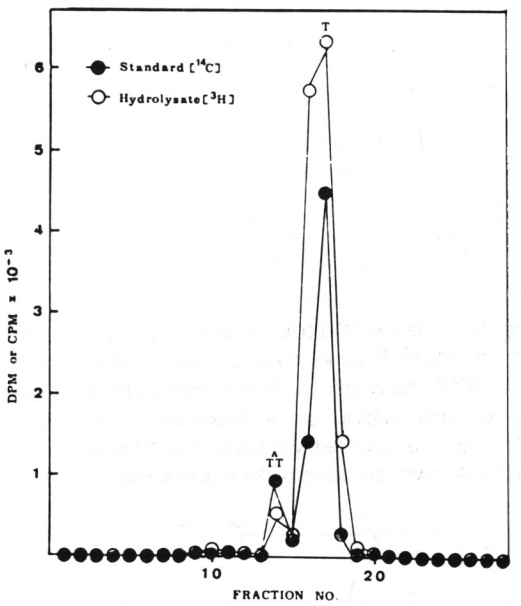

Fig. 4. HPLC of a hydrolysate of UV-irradiated *E. coli* [^{3}H]-thymidine incorporated *E. coli* DNA was UV-irradiated (50 J/m^2) to yield a thymine dimer and hydrolyzed at 175°C for 2 hours. Chromatography on Spherisorb C6 (5μm) was at 1ml/min, and 10 drop fractions were collected and then counted. Standard thymine dimer was prepared by UV-irradiation (500 J/m^2) of [^{14}C]- thymine and chromatographed in the same way.

Purification of AP DNA Endonucleases

DEAE Trisacryl gel column chromatography of AP DNA endonuclease extracted from rat liver chromatin is shown in Fig. 5. APcI was unbound to the gel, while APcII and APcIII (P_2) were bound to the gel and eluted out at 0.1M NaCl concentration. When the unbound fractions containing AP DNA endonuclease activity were pooled and chromatographed on a Sephadex G-150 column, a major single peak of AP DNA endonuclease of which molecular weight of approximately 30,000 daltons (APcI) was appeared (Fig. 6). When P_2 was subjected to Sephadex G-150 column chromatography, two enzyme peaks which have molecular weights of 41,000 (APcII) and 13,000 (APcIII) were appeared (Fig. 7). AP DNA cellulose affinity column chromatography of the pooled fraction

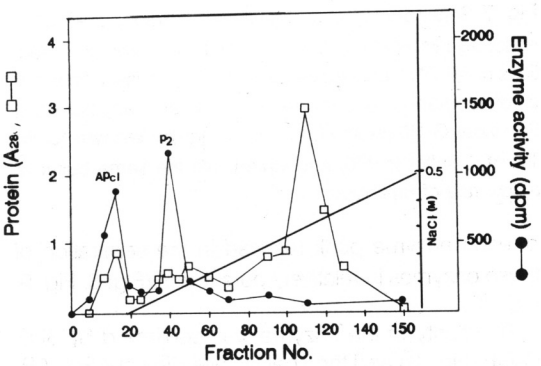

Fig. 5. DEAE-Trisacryl column chromatography of AP DNA endonucleases from rat liver chromatin. Chromatin extract was applied on a DEAE Trisacryl column (2.6×25cm) equilibrated with 50 mM Tris-HCl buffer (pH7.0) and eluted with the same buffer containing a linear gradient of 0-0.4M NaCl at a rate of 20ml/hour.

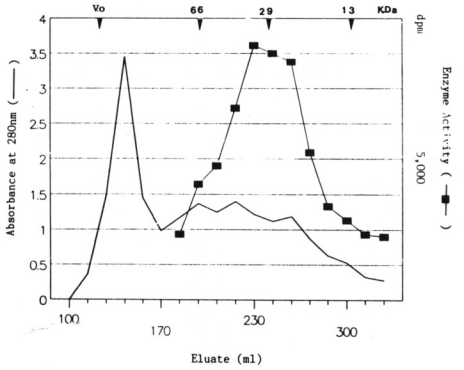

Fig. 6. Sephadex G-150 column chromatography of AP DNA endonuclease (APcI) from rat liver chromatin. Fractions of APcI from DEAE-Trisacryl column chromatography (unbound) were pooled and applied on a Sephadex G-150 column (2.6x102cm) equilibrated with 10mM Tris-HC1 buffer (pH7.0) and eluted with the same buffer at a flow rate of 8ml/hour.

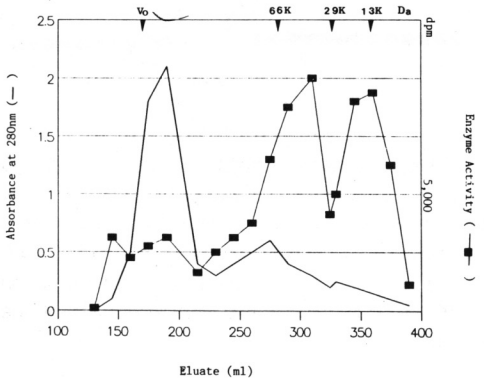

Fig. 7. Sephadex G-150 column chromatography of AP DNA endonuclease (APcII & APcIII) from rat liver chromatin. Fractions of AP DNA endonuclease from DEAE Trisacryl column chromatography (bound) were pooled and applied on a Sephadex G-150 column (2.6×102cm) equilibrated with 10mM Tris-HC1 buffer (pH7.0) and eluted with the same buffer at a flow rate of 8ml/hour.

of each enzyme peak resulted in the separation of these enzymes in relatively pure forms (Fig. 8, Fig. 9, Fig. 10).

The purity of the enzyme was confirmed by SDS PAGE (Fig. 11) and the specific activities of APcI, APcII and APcIII were 62.5, 83.3 and 52.0 EU/mg protein, respectively (Table 1).

Some Properties of Rat Liver Chromatin AP DNA Endonucleases

The molecular weights for APcI, APcII and APcIII were estimated from gel filtration (Sephadex G-150

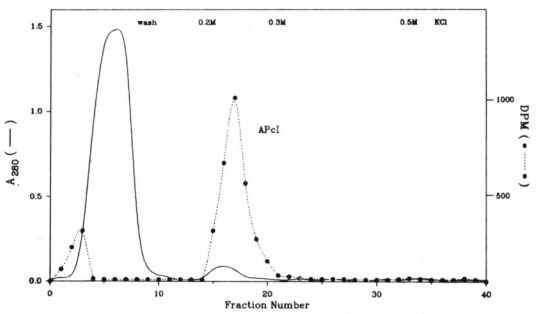

Fig. 8. AP DNA cellulose affinity column chromatography of AP DNA endonuclease (APcI) from rat liver chromatin. Fractions of AP DNA endonuclease (APcI) from Sephadex G-150 column chromatography were pooled and chromatographed With a AP DNA cellalose columa equilibrated with 20mM Tris-Hcl buffer)pH 8.1) containing 8.1mM EDTA, 0.4mM 2-ME and 10% geycerel, then eluted with a step wise gradient of 0, 0.2, 0.3, and 0.5M Kcl at a flow rate of 4ml/hour.

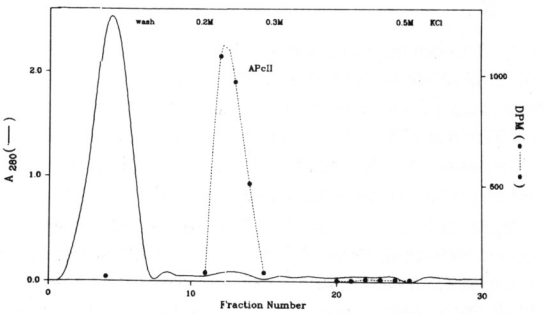

Fig. 9. AP DNA cellulose affinity column chromatography of AP DNA endonuclease (APcII) from rat liver chromatin. Fractions of AP DNA endonclease (APcII) from Sephadex G-150 column chromatography were pooled and applied on a AP DNA cellulose column equilibrated with 20mM Tris-HCl buffer (pH8.1) containing 0.1mM EDTA, 0.4mM 2-ME and 10% glycerol, then eluted with a stepwise gradient of 0, 0.2, 0.3, and 0.5M KCl at a flow rate of 4ml/hour.

column cromatography) and by SDS PAGE as 30,000, 42,000 and 13,000, respectively, indicating that all 3 enzymes consist of single polypeptide subunit (Fig. 6, 7 and 11, Table 2).

The isoelectric points of APcI, APcII and APcIII were estimated to be 7.2, 6.3 and 6.2 respectively.

DISCUSSION

The type of DNA damage is varied with the kind

Table 1. Purification of AP DNA endonuclease from rat liver chromatin

Step	Enzyme	Total activity (units)*	Protein (mg)	Specific activity (unit/mg protein)
Chromatin extract	APcI+APcII+APcIII	2,375.0	1,224.0	1.9
DEAE-Tris acryl	APcI (unbound)	703.0	63.3	11.1
	APcII+APcIII (bound)	1,068.0	107.8	9.9
Sephadex G-150	APcI	559.0	37.3	15.0
	APcII	272.0	12.6	21.6
	APcIII	205.8	8.3	24.8
AP DNA cellulose	APcI	75.0	1.2	62.5
	APcII	108.2	1.3	83.3
	APcIII	41.6	0.8	52.0

* A unit defined as p mole of AP site excised per min.

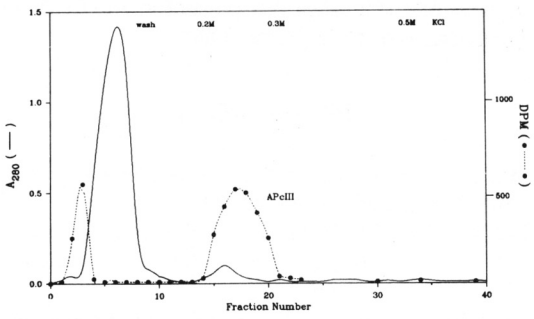

Fig. 10. AP DNA cellulose affinity column chromatography of AP DNA endonuclease (APcIII) from rat liver chromatin. Fractions of AP DNA endonuclease (APcIII) from Sephadex G-150 column chromatography were pooled and chromatographed as in Fig. 9.

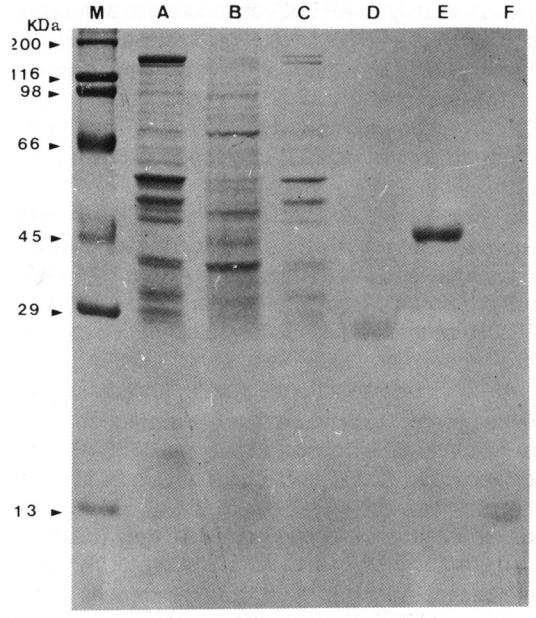

Fig. 11. SDS polyacrylamide gel electrophoresis of AP DNA endonucleases in rat liver chromatin. SDS polyacrylamide gel (12%) electrophoresis was run at a constant 30mA for 5 hrs and the gel was stained with Coomassie blue, and then was destained with 30% methanol containing 10% acetic acid. M, Molecular weight marker; A, Dialysate of chromatin extract; B, Unbound fractions of DEAE-Trisacryl chromatography; C, Bound fractions of DEAE-Trisacryl chromatography; D, Purified APcI; E, Purified APcII; F, Purified APcIII.

of damaging agent. Alkylating agents such as methylmethanesulfonate methylate DNA mainly at No. 3 position of adenine ring and a specific DNA N-glycosylases are responsible for the repair of alkylated DNA to generate AP DNA (Brent and Gallagher, 1983).

Irradiation of UV light to DNA causes the formation of cyclobutane type pyrimidine dimer. The dimer must be excised for repair of thymine dimer (Hanawalt et al., 1979).

It has been known that chemical hepatocarcinogens such as N-acetylaminofluorene (AAF) and 3'-Me DAB are metabolized to ultimate carcinogens that bind to the DNA and form DNA adducts (Lin et al., 1975, Labuc and Blunck, 1979).

But the mechanism on the repair of carcinogen-bound DNA adduct is not well known. It has been reported that the repair of AAF-DNA adduct was accomplished by a repair endonucleases (Van Lanker and Tonura, 1974). However, Lee and her associates (1986) reported that 3'-Me MAB DNA adduct formed in vitro did not subjected to depurination nor N-glycosylase-catalyzed depurination. In the present study 3 kinds of damaged DNA were prepared in ord-

Table 2. Some characteristics of AP DNA endonuclease purified from rat liver chromatin

Enzyme	Molecular weight (daltons)	No. subunit	Isoelectric point (pI)	Substrate reacted
APcI	30,000	1	7.2	AP DNA
APcII	42,000	1	6.3	AP DNA, UV DNA
APcIII	13,000	1	6.2	AP DNA, UV DNA
				AP DNA, UV DNA
				3-Me MAB DNA adduct

er to test the purified AP DNA endonucleases from rat liver chromatin for their substrate specificity.

In this study APcII and APcIII acted on both AP DNA and UV DNA as substrates, indicating that they possessed not only an AP DNA endonuclease activity but also pyrimidine dimer DNA N-glycosylase activity, or that they could be a multifunctional enzymes containing two functional units of AP DNA endonuclease and UV DNA endonuclease. However, APcI has been known to contain only AP DNA endonuclease (Lee, et al., 1986).

EU is generally adopted in the expression of most enzyme activity of AP DNA endonuclease, however, it was suggested that the number of AP site in AP DNA substrate should be determined for the calculation of AP DNA endonuclease activity (Goldmark and Linn, 1970: Tomkinson, 1988; Verly et al., 1981).

AP DNA prepared from *E.coli* chromosomal DNA in the present study had a number average molecular weight of 69.1kDa with an average of 58 AP sites per molecule (12.357 nucleotides) that is 29 times of that of T_7 phage AP DNA (6.46 AP sites/39.936 nucleotides) reported by Goffin and Verly (1984).

The kind of DNA and the condition for AP DNA preparation might result in great differences in the number of AP sites generated in DNA.

Three kinds of AP DNA endonucleases (APcI, APcII, APcIII) purified from rat liver chromatin differed in their molecular weights and isoelectric points in the present study.

The molecular weights of AP DNA endonuclease isolated from human fibroblast (Mosbaugh and Linn, 1980), lymphoblasts (Brebt, 1976) and human placenta (Linsley et al., 1977) have been reported as 25,000—40,000, and the presence of 2 different AP DNA endonucleases in human fibroblasts and 6 different isoenzyme in human placenta have also been reported. The MW of APcII (MW 42,000) is close to the molecular weight (39,000) of AP DNA endonuclease isolated from rat liver chromatin by Cesar and Verly (1983), and the MW of APcIII (13,000) is very close to that (MW 12,500) isolated from rat liver nuclei by Thibodeau et al. (1980).

However, the molceular weight of APcI (30,000) is not similar to any of them reported previously.

The differences in their molecular weights and iso electric points implicates that various functional as well as structural diversities are present in AP DNA en donucleases in rat liver chromatin.

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