ISOLATION AND PURIFICATION OF CALCIUM AND MAGNESIUM DEPENDENT ENDONUCLEASE FROM RAT LIVER NUCLEI

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SUMMARY: An endonuclease associated with rat liver chromatin was extracted with 0.6 M NaCl and purified by ammonium sulfate fractionation and Sephadex G-100 gel filtration. The enzyme produces single strand scissions on native DNA at neutral pH in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$. Alkali-denatured DNA was not nicked by the enzyme. Omission of Ca^{2+} reduced the enzyme activity to about one seventh. Without Ca^{2+}, however, Mn^{2+} was more effective than Mg^{2+}. The molecular weight of the enzyme is about 27,000.

INTRODUCTION

Burgoyne et al. (1) reported that DNA synthesis in isolated rat liver nuclei was stimulated in the presence of $CaCl_2$. They suggested participation of a Ca^{2+}-dependent endonuclease (2) in the stimulation of DNA synthesis by Ca^{2+}. However, such Ca^{2+}-dependent DNase has not been isolated and purified. Moreover, several kinds of DNase, reported to be present in rat liver (3-6), were inhibited by $CaCl_2$. In this communication, we report the isolation and purification of an endonuclease associated with rat liver chromatin, that is stimulated by $CaCl_2$ in the presence of $MgCl_2$.

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*Abbreviations: EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol Bis-(β-aminoethylether)-N, N'-tetraacetate; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Isolation of rat liver nuclei. The modified method of Burgoyne et al. (1) was used. The rat liver was homogenized in 0.34 M sucrose-solution A (1) containing 1 mM EDTA and 0.5 mM EGTA. The nuclei were separated by centrifugation on 1.37 M sucrose-solution A and then 2.2 M sucrose-solution A excluding EDTA and EGTA.

Extraction and purification of DNase. Isolated nuclei were suspended in a series of solutions containing various concentrations of NaCl and 1 mM 2-mercaptoethanol. The nuclear suspension was sonicated for 1 min with a Branson sonifier, stirred for 120 min at 0^{o}C and centrifuged for 14 hours at 105,000×g. The supernatant was dialysed against TM buffer (0.02 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol). After removal of the precipitate, solid ammonium sulfate was added to 40 % saturation and the suspension was centrifuged. The supernatant was dialysed against TM buffer containing 15 % ethyleneglycol and further fractionated by Sephadex G-100 gel filtration (ref. the legend to Fig. 2).

Preparation of labeled E. coli DNA. Tritium-labeled DNA was obtained by SDS-phenol method (7) from E. coli B_3 the DNA of which was labeled by incubation of the bacteris in a synthetic medium containing ^{3}H-thymidine (2 μC and 1 μg/ml).

Assay of DNase activity. DNase activity was determined by counting acid-soluble radioactivity after incubation of labeled E. coli DNA (27 μg, 80,000 cpm) for 40 min in a final volume of 0.5 ml including 10 μmoles Tris-HCl, pH 7.4, 2.5 μmoles $MgCl_2$ and enzyme protein (the standard assay mixture).

Alkaline sucrose gradient centrifugation. An aliquot of the sample was layered on 4.8 ml of 5-20 % alkaline sucrose gradient containing 0.3 M NaOH, 0.7 M NaCl and 50 mM EDTA, and centrifuged at

34,000 rpm in the Hitachi RPS 40 rotor for 4 hours at 10°C. The gradient was fractionated into scintillation vials and the radioactivity was counted as described in the legend of Table 1.

RESULTS

It was not possible to solubilize a DNase stimulated by Ca^{2+} in the presence of Mg^{2+} (Ca-Mg DNase) from nuclei with TM buffer containing 0.14 M NaCl, suggesting an association of the enzyme with chromatin. Fig. 1 shows that the enzyme was partially

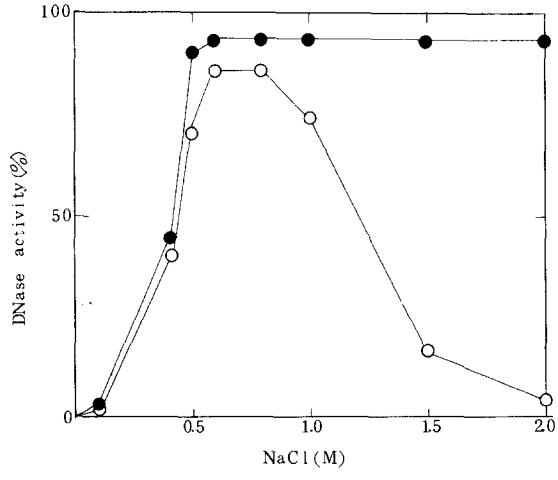

Fig. 1. NaCl extraction of the Ca-Mg DNase from rat liver nuclei. Total nuclear DNase activity (100 %) represents the summation of the extracted activity after overnight centrifugation of nuclei in the NaCl solution and the unextracted activity remained in the nuclei. ●—● , the enzyme activity extracted with NaCl; ○—○ , the enzyme activity remained in the supernatant after dialysis of the NaCl extract.

released from nuclei with 0.4 M NaCl and more than 90 % of the enzyme was extracted with 0.6 M NaCl. However, with NaCl concentrations exceeding 0.8 M, the extracted enzyme formed an insoluble precipitate after dialysis against TM buffer. All of the enzyme

activity extracted with 2 M NaCl precipitated after the dialysis.

To remove other contaminating DNases from the extract, the 0.6 M NaCl extract was fractionated with ammonium sulfate. As shown in Table 1, 70 % of the Ca-Mg DNase in the 0.6 M NaCl ex-

Table 1. Separation of the Ca-Mg DNase by 40 % ammonium sulfate

Condition	Deoxyribonuclease activity (cpm)		
	0.6 M NaCl extract	Supernatant (40 %)	Precipitate (40 %)
Native DNA	7,550	1,610	3,350
Native DNA +1 mM Ca^{++}	14,390	9,400	2,850
Denatured DNA	9,780	2,520	8,870

The DNase activity was assayed under various conditions in the presence of 5 mM $MgCl_2$. The reaction was stopped by 5 % trichloroacetic acid. The suspension was centrifuged at 3,000 rpm for 10 min and 0.2 ml of the supernatant was counted in 10 ml of dioxane scintillator (40 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml methanol and 20 g ethyleneglycol in 1,000 ml dioxane).

tract was recovered in the supernatant of the 40 % ammonium sulfate fractionation. The enzyme in the supernatant was stimulated about six fold on addition of 1 mM $CaCl_2$. The optimum pH under these conditions was about 7. On the other hand, the DNase in the 40 % ammonium sulfate precipitate was inhibited by 1 mM $CaCl_2$ and preferentially hydrolyzed heat denatured DNA. The enzyme in the 40 % amminium sulfate precipitate resembles the DNase reported by Howk and Wang (8) and O'Connor (9).

For further purification, the Ca-Mg DNase in the 40 % ammo-

nium sulfate supernatant was chromatographed on a Sephadex G-100 gel column. Unexpectedly, however, no enzyme capable of degrading native DNA to an acid soluble product was detected. This complete disappearance of activity suggested that a factor was separated that promoted formation of acid soluble products. Thus, the DNase activity of each fraction of the Sephadex column was assayed in the presence of an exonuclease, partially purified from rat ascites hepatoma (AH130) nuclei, that degrades short fragments of DNA (10). Fig. 2 demonstrates the elution of the Ca-Mg DNase

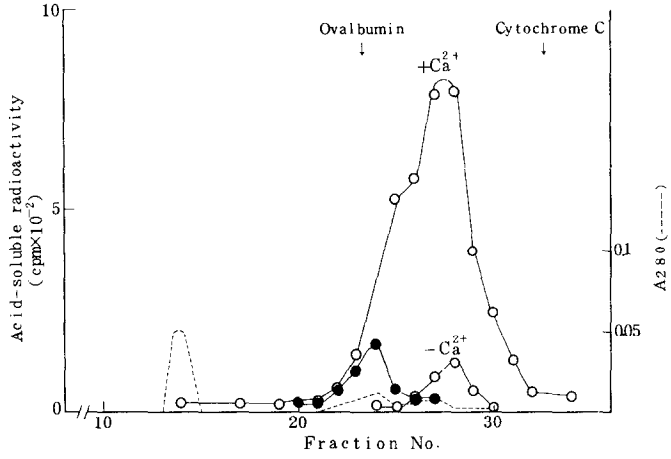

Fig. 2. Sephadex G-100 column chromatography of the 40 % ammonium sulfate supernatant. The dialysed supernatant (5 ml) was charged on to a Sephadex G-100 column (3 × 38 cm) previously washed with TM buffer containing 15 % ethyleneglycol and eluted with the same buffer. 5 ml fractions were collected and 0.1 ml aliquots were assayed for DNase activity as described in the legend to Table 1. O—O , degradation of native DNA in the presence of AH130 exonuclease with and without added 1 mM $CaCl_2$; ●—● , degradation of heat denatured DNA in the absence of AH130 exonuclease and Ca^{2+}.

activity between ovalbumin and cytochrome C and the molecular weight of the enzyme was calculated about 27,000. Moreover, this appearance of the Ca-Mg DNase was also observed with the

addition of an aliquot of the 40 % ammonium sulfate precipitate. A small amount of DNase activity, that degrades heat denatured DNA and thus resembles the DNase in the 40 % ammonium sulfate precipitate, separately emerged before the Ca-Mg DNase on the Sephadex column (Fig. 2). From these results it is concluded that formation of acid soluble products from DNA was required collaboration of the Ca-Mg DNase and an exonucleolytic enzyme remained in the 40 % ammonium sulfate supernatant.

To confirm that the Ca-Mg DNase is an endonuclease, E. coli DNA was incubated with an aliquot of the peak fraction of the Sephadex G-100 column chromatography. The digested DNA was centrifuged on alkaline sucrose gradient. Single strand scission of native DNA, observed with 5 mM Mg^{2+} only, was greatly stimulated by the addition of 1 mM $CaCl_2$. Without Ca^{2+}, however, it was found that Mn^{2+} was more effective than Mg^{2+} (Fig. 3A). Alkali-denatured DNA was not attacked by this enzyme in the presence or absence of $CaCl_2$ (Fig. 3B).

DISCUSSION

The evidence that the Ca-Mg DNase begins to be released from nuclei with 0.4 M NaCl indicates that the endonuclease is tightly bound to chromatin, and we could not detect its activity in the cytoplasm. Rat liver nuclei have been reported to contain two kinds of DNases. One prefers denatured DNA (8,9) and the other, associated with chromatin, degrades native DNA (9). However, the latter enzyme is different from the Ca-Mg DNase reported in this communication because it is more active with Mg^{2+} than with Mn^{2+} and inhibited by Ca^{2+}.

Furthermore, the Ca-Mg DNase differs from the DNases detected in the soluble fraction (4), in lysosomes (11), in mitochondria

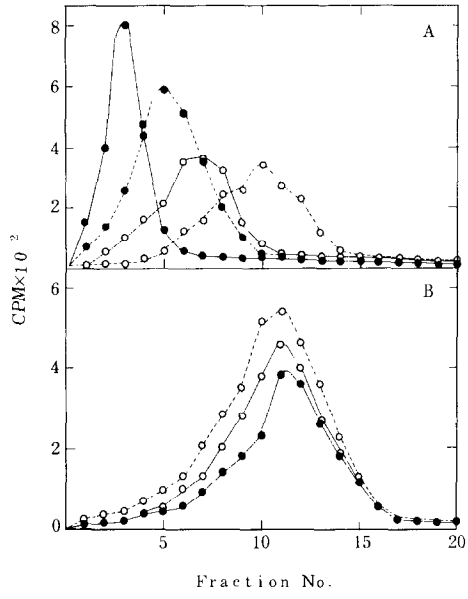

Fig. 3. Sucrose gradient centrifugation of the Ca-Mg DNase-treated E. coli DNA. Native (A) or alkali-denatured DNA (B) was incubated with 0.1 ml of the peak fraction of Sephadex G-100 column chromatography (Fig. 2, fraction No. 29) for 30 min in the standard assay mixture. The reaction mixture was made alkaline with 0.3 M KOH and 0.1 M EDTA. The sample was centrifuged on alkaline sucrose gradient as described in "MATERIALS AND METHODS". Additions: ○—○ , none; ●—● , plus 1 mM $CaCl_2$; ●---● , plus 1 mM $MnCl_2$ minus $MgCl_2$; ○---○ , without the enzyme.

(5) and in ribosomes (6), in its substrate specificity, metal requirement and pH optimum.

DNase I and a very similar enzyme present in Paracentrotus lividus embryos (12) are synergistically stimulated by Ca^{2+} and Mg^{2+}. However, unlike from the enzyme reported in this communication, both enzymes attack denatured DNA.

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