BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 133–138 (1997)
ARTICLE NO. RC976362

Mg^{2+}- or Mn^{2+}-Dependent Endonuclease Activities of Human Myeloid Leukemia Cells Capable of Producing Nucleosomal-Size DNA Fragmentation

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Received February 24, 1997

The presence of at least two distinct Mg^{2+}- or Mn^{2+}-dependent, Ca^{2+}-independent endonuclease activities was shown in the myeloid leukemia cell line P39. One of them was recovered from nuclear extracts and the other from a cytoplasmic fraction. The molecular size of the former was 30 kDa in both gel filtration and activity gel and that of the latter approximately 130-140 kDa in gel filtration and 65-70 kDa in activity gel. These two activities were almost completely inhibited by 0.1 mM $ZnCl_2$ or 0.1 mM aurintricarboxylic acid, common inhibitors of apoptosis. Both could produce nucleosomal-size DNA fragmentation when incubated with diethyl-pyrocarbonate-treated nuclei as substrates, and the pattern of cleavage was 3′-OH and 5′-P. Taken together, either or both of these activities may be associated with apoptosis of myeloid leukemia cells.

Apoptosis, which was originally described by Kerr et al. in 1972, has become one of the major topics in a variety of biological and pathological settings [1]. Nucleosomal-size DNA fragmentation has been recognized as a terminal event as well as a hallmark of apoptosis [2].

Several endonucleases have been proposed to be associated with the nucleosomal-size DNA fragmentation to date. Ca^{2+}/Mg^{2+}-dependent endonucleases in murine thymocytes such as NUC18/cyclophilin A, DNase I and DNase γ represent one such example [3-6]. On the other hand, Barry and Eastman suggested that DNase II, a divalent cation independent acidic endonuclease, might induce typical DNA fragmentation in apoptosis in association with intracellular acidification [7]. Thus, identity of endonuclease(s) involved in apoptosis is still controversial due largely to the lack of direct evidence of the participation in apoptosis.

We have previously reported that the nuclei of intact P39 cells, a human myeloid cell line, contain Mg^{2+}-dependent, but Ca^{2+}-independent endonuclease activity capable of inducing nucleosomal-size DNA fragmentation [8]. However, Matsubara, et al. reported that this type of endonuclease was expressed in only the cytosol but not in the nuclei of myeloid leukemia cells [9]. In this report, we partially purified this type of activit ies from both the nuclear and the cytoplasmic fractions, separately. Throughout the purification, we evaluated the endonuclease activities by the ability to induce nucleosomal-size DNA fragmentation. Using these semi-purified samples, we further examined biochemical properties of these activities.

EXPERIMENTAL PROCEDURES

Reagents, cell lines, and chromatographic media. All chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan) except where otherwise indicated. Jurkat and P39 [10] cell lines were obtained from Japanese Cancer Research Resources Bank. Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. For all experiments, exponentially growing cells were used. Econo-Pac S cartridges, Bio-Scale CHT2 and Bio-Silect SEC 250-5 columns were obtained from Bio-Rad Laboratories (Hercules, CA). HiTrap Heparin columns were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). These columns were used with a BioLogic System (Bio-Rad).

Assay for endonuclease activity specific for nucleosomal-size DNA fragmentation. DNase activity which can induce nucleosomal-size DNA fragmentation was measured according to the "target nuclear digestion" method [11]. In brief, we prepared Jurkat cell nuclei, endogenous endonuclease of which had been substantially inactivated by pretreatment with diethylpyrocarbonate (DEPC). Each sample

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Abbreviations used: DEPC, diethylpyrocarbonate; EGTA, ethyleneglycol-bis-tetraacetic acid; 2ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; NP-40, nonidet P-40; PAGE, polyacrylamide gel electrophoresis; CIP, calf intestinal alkaline phosphatase.

0006-291X/97 $25.00

was incubated with approximately 1×10^6 of these nuclei in 10 mM Tris-HCl pH 7.6, 1 mM $MgCl_2$, 2 mM ethyleneglycol-bis-tetraacetic acid (EGTA) and 1 mM dithiothreitol (Buffer A) for 16 h at 37°C. After the incubation, total DNA was extracted and electrophoresed in 1.5% agarose gel. Then the gel was stained with ethidium bromide followed by visualizing under UV light.

Subcellular fractionation. All steps were performed at 0-4°C. Approximately 2×10^9 P39 cells were washed 3 times with phosphate-buffered saline and suspended in 10 ml of 10 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$, 25 mM KCl, 1 mM 2-mercaptoethanol (2ME) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) (TKM buffer). The cell suspension was homogenized with a Dounce homogenizer for 100 strokes on ice. The resultant cytoplasm-free nuclei were examined by an inverted microscopy for the purity. Then equal volume of 0.6 M sucrose in TKM buffer was added to the homogenate, and the mixture was centrifuged at 700 × g for 15 min. The supernatant was mixed with 2% volume of 10% Nonidet P-40 (NP-40), shaken, centrifuged at 180,000 × g for 1 hr, and the supernatant was defined as the "cytoplasmic fraction". The pellet of 700 × g centrifugation was washed with 0.2% NP-40 in TKM buffer, resuspended in 0.6 M NaCl in TKM Buffer, shaken for 1 hr, centrifuged at 180,000 × g for 1 hr, and the supernatant was defined as the "nuclear extracts". Both the nuclear extracts and the cytosolic fraction were dialyzed against 10 mM Tris-HCl, pH 7.6, 1 mM 2ME, 0.2 mM PMSF, 25 mM NaCl and 20% ethyleneglycol, and the dialyzates were clarified by centrifugation at 180,000 × g for 30 min.

Partial purification of a nuclear endonuclease activity. The nuclear extracts from P39 cells were applied to a Econo-Pac S (cation exchanger) cartridge which had been equilibrated with 10 mM Tris-HCl, pH 7.6, 25 mM NaCl, 1 mM 2ME, 0.2 mM PMSF and 10% ethyleneglycol (Buffer B). Material was eluted with a linear gradient of 0.025 M to 0.3 M NaCl in Buffer B, and the active fractions eluted with NaCl ranging from 0.12 to 0.22 M were pooled (Fraction Nuc-I). Fraction Nuc-I was applied to a HiTrap Heparin (affinity) column and was eluted with a linear gradient of 0.1 M to 0.6 M NaCl in Buffer B. The active fractions eluted with 0.31 to 0.43 M NaCl were pooled (Fraction Nuc-II). Fraction Nuc-II was concentrated with an ultrafiltration tube, then applied to a Bio-Silect SEC 250-5 (gel filtration) column which had been equilibrated with 50 mM Na-Phosphate, pH 7 and 150 mM NaCl. Material was eluted with the same buffer, and the active fractions were collected (Fraction Nuc-III, Fig. 1. Panel a). The activity of this fraction was relatively stable and was used for biochemical characterization. Alternatively, Fraction Nuc-II was dialyzed against 10 mM Na-Phosphate, pH 6.8, 25 mM NaCl, 0.2 mM PMSF and 1 mM 2ME and was applied to a Bio-Scale CHT2 (hydroxylapatite) column which had been equilibrated with 10 mM Na-Phosphate, pH 6.8 and 25 mM NaCl. Material was eluted with a linear gradient of 0.01 M to 0.3 M Na-Phosphate and the active fractions eluted with 0.11 M to 0.17 M Na-Phosphate were pooled (Fraction Nuc-IV). Fraction Nuc-IV was applied to a Bio-Silect SEC 250-5 column, and the active fractions (Fraction Nuc-V) were used for a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Partial purification of a cytoplasmic endonuclease activity. The cytoplasmic fraction was applied to a BioScale Q-2 (anion exchanger) column which had been equilibrated with 10 mM Tris-HCl, pH 7, and was eluted with a linear gradient of 0.025 M to 0.3 M NaCl. Active fractions eluted with 0.11 to 0.18 M NaCl were collected (Fraction Cyt-I). Fraction Cyt-I was applied to a HiTrap Heparin column and was eluted with a linear gradient of 0.1 M to 0.6 M NaCl in Buffer B. The active fractions eluted with 0.3 to 0.39 M NaCl were pooled (Fraction Cyt-II). Fraction Cyt-II was concentrated and applied to a Bio-Silect SEC 250-5 column, and the active fractions were collected (Fraction Cyt-IIIa and Cyt-IIIb, Fig. 1. Panel b). The activity of Fraction IIIa was relatively stable and was used for biochemical characterization. Alternatively, Fraction Cyt-II was dialyzed against 10 mM Na-Phosphate, pH 6.8, 25 mM NaCl, 0.2 mM PMSF and 1 mM 2ME and was applied to a Bio-Scale CHT-2 column which had been equilibrated with 10 mM Na-Phosphate, pH 6.8 and 25 mM NaCl. Material was eluted with a linear gradient of 0.01 M to 0.3 M Na-Phosphate and the active fractions eluted with 0.21 M to 0.24 M Na-Phosphate were pooled (Fraction Cyt-IV). Fraction Cyt-IV was applied to a Bio-Silect SEC 250-5 column, and the active fractions corresponding to the size of Fraction IIIa were applied to SDS-PAGE (Fraction Cyt-Va).

Enzyme purity. The purity of nuclease samples was examined by SDS-PAGE followed by staining with silver.

Activity gel analysis. We detected Mg^{2+}-dependent DNase activities using activity gel method [12] with some modifications. Briefly, samples were subjected to polyacrylamide gel containing 0.1% SDS, 10 μg/ml bovine serum albumin and 30 μg/ml heat-denatured calf thymus DNA (SIGMA, St. Louis, MO), and electrophoresed at 4°C. After the electrophoresis, the gel was incubated in 10 mM Tris, pH 7.6, 0.2 mM PMSF, 1 mM 2ME and 0.2% NP-40 at 4°C for 1 h to remove SDS. This incubation was repeated 3 times, then the gel was incubated in 10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM 2ME and 0.2% NP-40 at 37°C for 24-72 h followed by staining with ethidium bromide.

Endonuclease activity against plasmid DNA. Fifty nano gram plasmid DNA (pHSG-336, from TAKARA, Otsu, Japan) was incubated with nuclease samples in 10 mM Tris-HCl, pH 7.6 at 37°C for 30 min in the presence of various concentrations of $MgCl_2$, $CaCl_2$ or $MnCl_2$. After inactivation of endonucleases by 30 min incubation at 55°C with 25 mM EDTA and 2 mg/ml proteinase K, the samples were electrophoresed in 0.8% agarose gel followed by visualization under UV light.

Terminal nucleotide analysis. An analysis for cleavage terminals of DNA fragments was performed according to the method of Shiokawa, et al. with some modification [13]. Briefly, the 5′-OH ends of the DNA fragments were labeled with [γ-^{32}P]ATP by incubating with polynucleotide kinase (TOYOBO, Osaka, Japan), and was spread in 1.5% agarose gel. Then the labeled DNA was transferred to nylon membranes, and the membranes were autoradiographed. On the other hand, the 3′-OH ends of the DNA fragments were labeled with digoxigenin-ddUTP by incubating with terminal transferase using DIG Oligonucleotide 3′-End Labeling Kit (Boehringer Mannheim, Mannheim, Germany). The labeled DNA was spread in 1.5% agarose gel and transferred to nylon membranes, and was detected using Nucleic Acid Detection Kit (Boehringer Mannheim). Before each of the end labeling experiments, an aliquot of each DNA sample was incubated with 2 U of calf intestinal alkaline phosphatase (CIP, TOYOBO) in order to hydrolyze the phosphoryl groups of the ends of DNA fragments, and was used as a positive control.

RESULTS AND DISCUSSION

With liquid chromatographical procedures, we partially purified endodeoxyribonuclease activities which could induce nucleosomal size-DNA fragmentation in the presence of Mg^{2+} and EGTA from the nuclear extracts and the cytoplasmic fraction of P39 cells. For this purpose, we measured the activity of endonuclease on DEPC-treated Jurkat cell nuclei throughout the procedures. The molecular size of the endonuclease activity from the nuclear extracts estimated by gel filtration was approximately 30 kDa (Fig. 1. Panel a, Fraction Nuc-III). In gel filtration assay of the cytoplasmic fraction, two peaks of nuclease activity were observed; a major peak at approximately 130-140 kDa (Fig. 1. Panel b, Fraction Cyt-IIIa) and a minor peak at 30

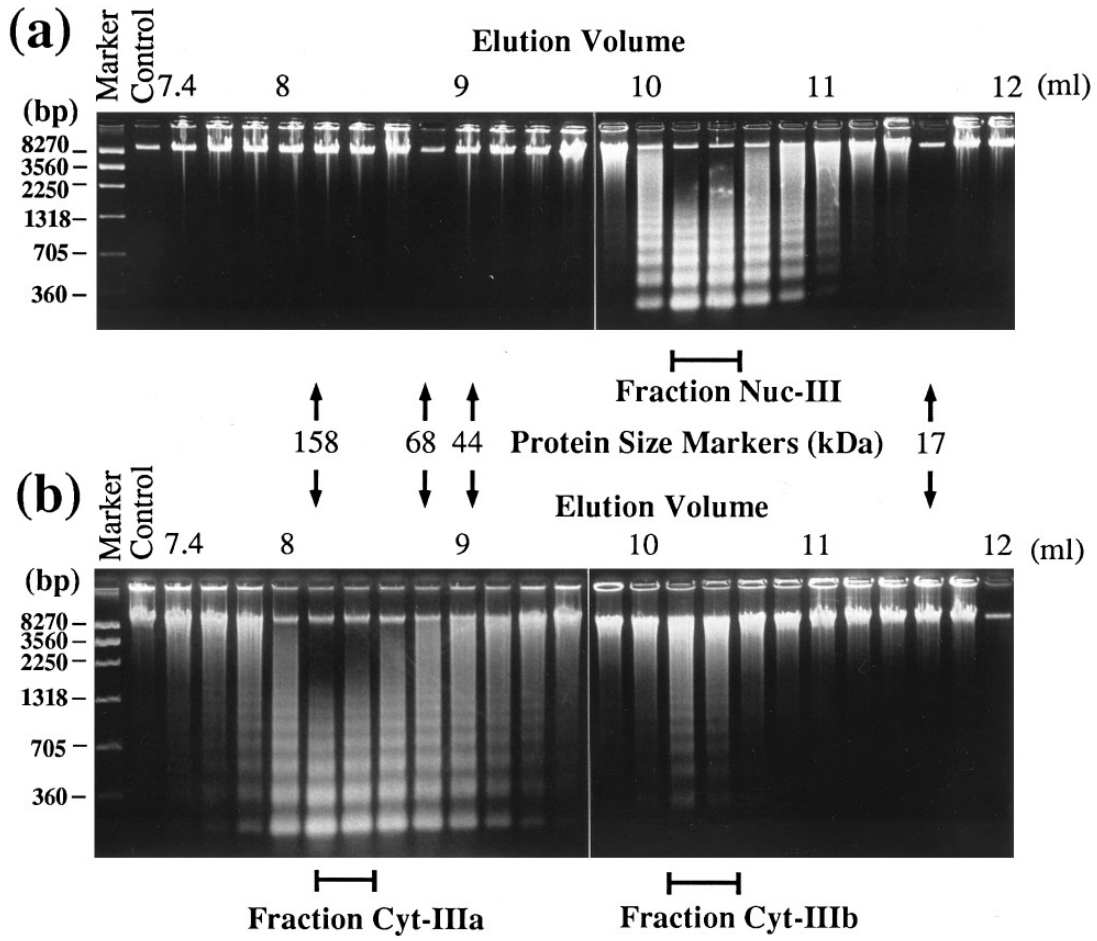

FIG. 1. The estimation of the molecular size of endonuclease activities by gel filtration. (a) After sequential liquid chromatographic procedures using an Econo-Pac S (cation exchanger) cartridge and a HiTrap Heparin (affinity) column, the partially purified endonuclease activity from the nuclear extracts was applied to a Bio-Silect SEC 250-5 (gel filtration) column and the active fractions were collected (Fraction Nuc-III). (b) The endonuclease activity was also partially purified from the cytoplasmic fraction using BioScale Q-2 (anion exchanger) and HiTrap Heparin columns and then applied to a Bio-Silect SEC 250-5 column. A major and a minor peak of the activity were collected (Fraction Cyt-IIIa and Cyt-IIIb, respectively). Mg^{2+}-dependent, Ca^{2+}-independent endonuclease activity of each tube was evaluated by the ability to cleave DEPC-treated nuclei in the presence of 1 mM Mg^{2+} and 2 mM EGTA (see the Experimental Procedures for details). DNA extracted from the DEPC-treated nuclei incubated in the same buffer without adding any nuclease samples was used as a control. The size marker proteins used were bovine IgG (158 kDa), bovine serum albumin (68 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17.0 kDa).

kDa (Fraction Cyt-IIIb). According to the SDS-PAGE analysis of Fraction Nuc-V, there was a major band at an approximate size of 30 kDa together with some minor bands near by (Fig. 2. Panel a). For Fraction Cyt-Va, there were two major bands in the approximate sizes of 58 kDa and 66 kDa, with some minor bands (Fig. 2. Panel a). In the activity gel analysis, we used nuclear extracts and the cytoplasmic fraction because of the instability of semipurified samples. After 72 hr incubation in the presence of 1 mM $MgCl_2$, a band representing DNase activity appeared at an approximate size of 30 kDa in the lane of the nuclear extracts. For the cytoplasmic fraction, a clear band at an approximate size of 65-70 kDa was seen after 24 h incubation (Fig. 2. Panel b). These sizes were consistent with the sizes of the major band of Fraction Nuc-V and one of the major bands of Fraction Cyt-Va appearing in SDS-PAGE. The molecular size of the major cytoplasmic activity (Fraction Cyt-IIIa) estimated by gel filtration was approximately double the size estimated by activity gel, which may suggest its homodimeric structure.

The biochemical characteristics of the nuclear endonuclease activity (Fraction Nuc-III) and the major cytoplasmic endonuclease activity (Fraction Cyt-IIIa) were quite similar. Both activities were active at neutral pH, and were almost completely inhibited by 0.1 mM $ZnCl_2$, 0.1 mM aurintricarboxylic acid, 100 mM NaCl or 100 mM KCl (Fig. 3, panel a). To examine the divalent cation requirements of these activities, we used plasmid DNA as a substrate instead of the DEPC-treated

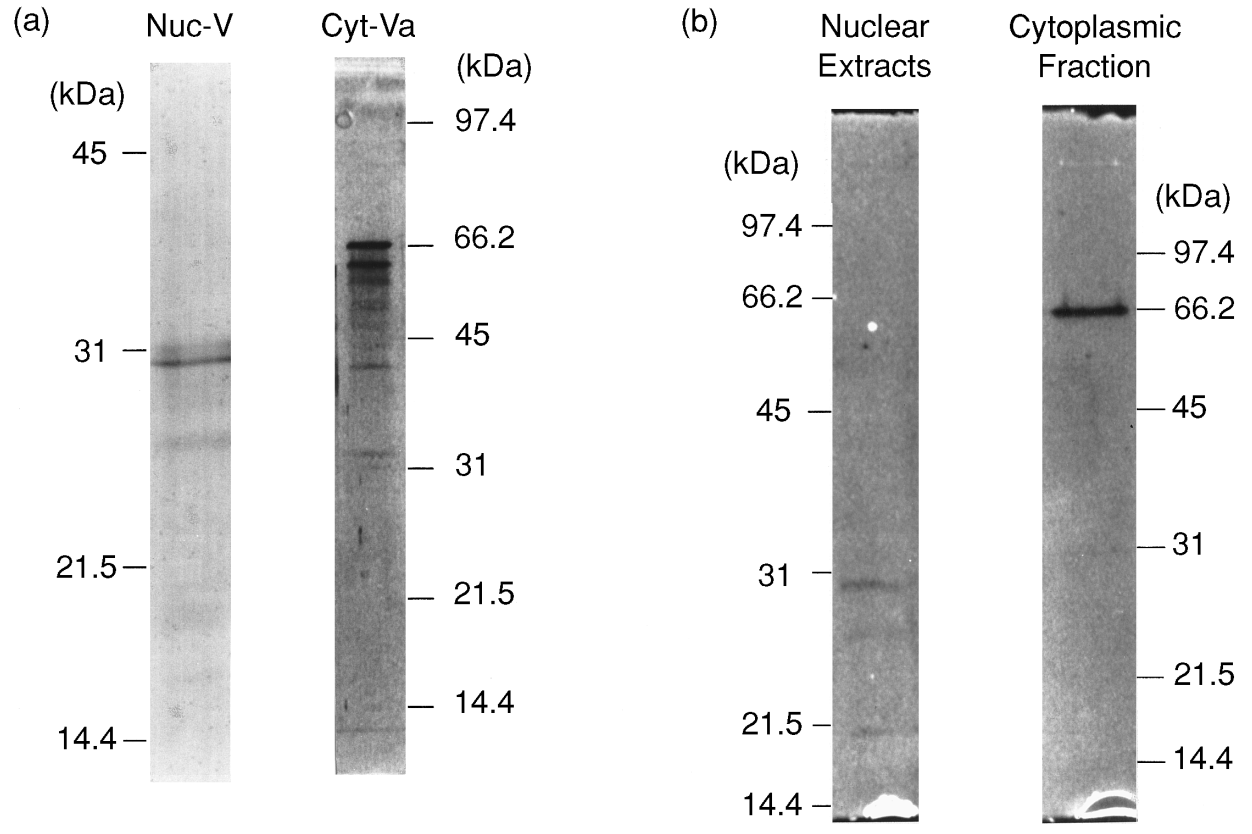

FIG. 2. (a) SDS-PAGE of the partially purified Mg^{2+}-dependent endonuclease activities from the nuclear extracts and the cytoplasmic fraction. Heat-denatured Fraction Nuc-V and Cyt-Va were electrophoresed in a 15 and 11% acrylamide slab gel, respectively, containing 0.1% SDS. The gel was stained with silver. (b) The activity gel analysis. The nuclear extracts and the cytoplasmic fraction of P39 cells were subjected to 11% polyacrylamide gel containing 0.1% SDS, 10 μg/ml bovine serum albumin, and 30 μg/ml heat-denatured calf thymus DNA, then electrophoresed at 4°C. After removing SDS, the gel was incubated in 10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1 mM 2ME, and 0.2% NP-40 for 24 h (the cytoplasmic fraction) or 72 h (the nuclear extracts) at 37°C. Then the gel was stained with ethidium bromide, and the bands representing DNase activity were visualized under UV light. The positions and the sizes of the marker proteins run in the same gel are as indicated.

nuclei because of the difficulty of complete removal of Mg^{2+} from the latter. Thirty min incubation of Fraction Nuc-III and Cyt-IIIa in the presence of either $MgCl_2$ or $MnCl_2$ resulted in a decrease in spherical form and an increase in open circular and linear form of plasmid DNA (Figure 3, panel b). These activities did not require Ca^{2+} ion, but were apparently inhibited by 10 mM $CaCl_2$. For the terminal nucleotide analysis, we prepared fragmented DNA extracted from the DEPC-treated Jurkat cell nuclei which had been digested by Fraction Nuc III or Fraction Cyt IIIa in buffer A for 16 h at 37°C. CIP-treated DNA was used as a positive control for each sample. In Fig. 3, Panel c, neither of the 5′ ends of the DNA fragments digested by Fraction Nuc-III or Cyt-IIIa were labeled with polynucleotide kinase. In contrast, the 3′ ends of these DNA fragments were labeled with terminal transferase. These results indicate that the pattern of the DNA fragments produced by the nuclear and cytoplasmic endonuclease activities we semi-purified were 3′-OH and 5′-P, which is known to be the pattern in apoptosis [13]. Though these results do not provide direct evidence for participation in apoptosis of the endonuclease activities we partially purified, these characteristics were consistent with their role in apoptosis. The actual functions of these activities in the viable or apoptotic cells remain to be delineated.

It is widely held that a Ca^{2+}/Mg^{2+}-dependent endonuclease is involved in the formation of nucleosomal-size DNA fragmentation in apoptosis. Thus an increase of intracellular Ca^{2+} during thymocyte apoptosis has been thought to be a trigger of the activation of this type of endonucleases [14, 15]. However, this notion is still controversial at least in myeloid cells. Intracellular Ca^{2+} concentration of HL60 cells was not elevated prior to DNA fragmentation induced by various stimuli including UV treatment [16]. In addition, intracellular Ca^{2+} elevation did not induce apoptosis but rather prolonged lifespan of neutrophils *in vitro*, and bis-(*o*-aminophenoxy)-N,N,N,N′-tetraacetic acid acetoxy-

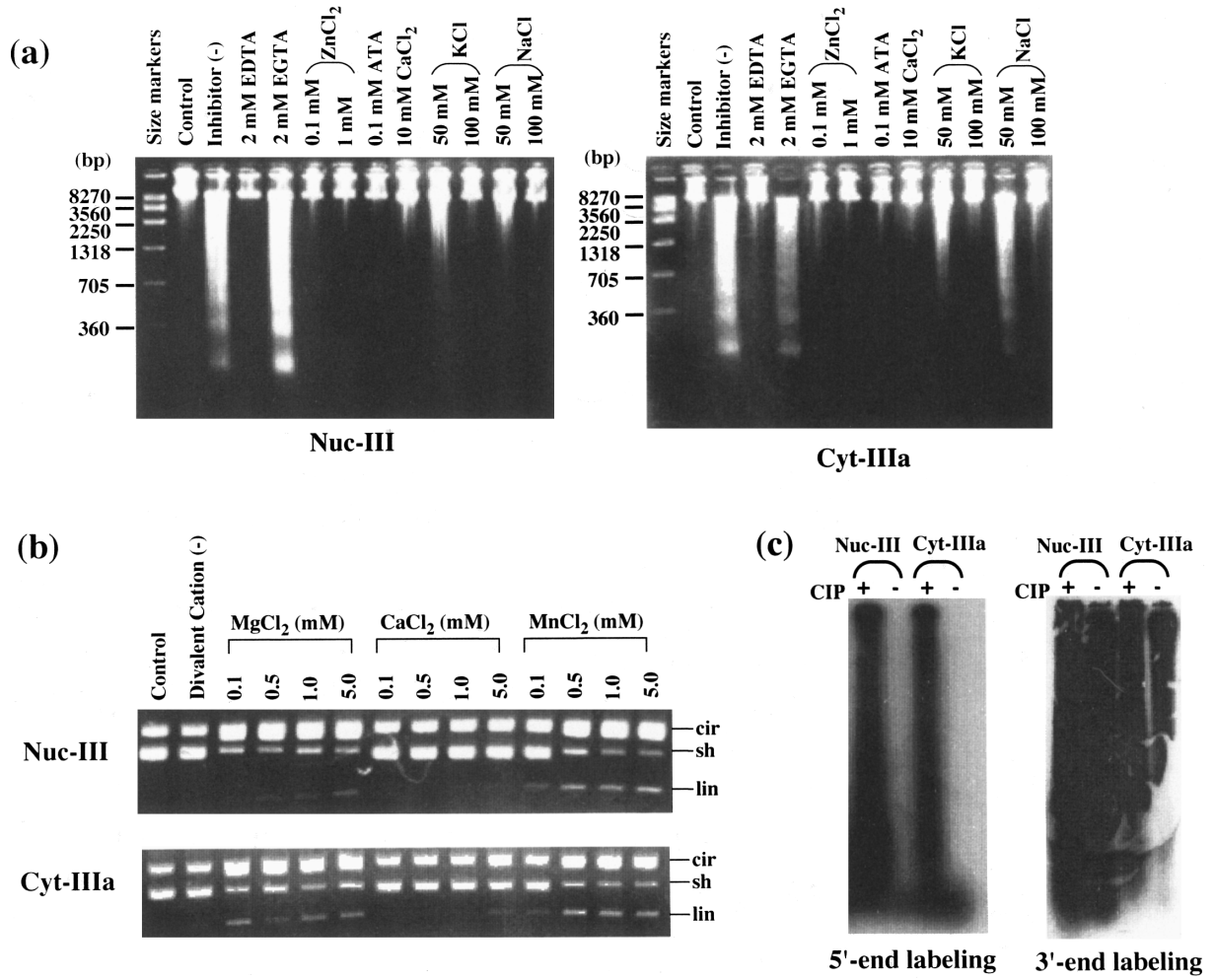

FIG. 3. (a) DEPC-treated nuclei were incubated with Fraction Nuc-III or Cyt-IIIa in 10 mM Tris-HCl, pH 7.6, and 1 mM $MgCl_2$ for 16 h at 37°C in the presence of indicated concentrations of EDTA, EGTA, $ZnCl_2$, ATA, NaCl, or KCl. Then the DNA was electrophoresed in 1.5% agarose gel. DNA extracted from the DEPC-treated nuclei incubated in 10 mM Tris-HCl and 1 mM $MgCl_2$ without adding any nuclease samples was used as a control. (b) Plasmid DNA (pHSG-336) was incubated with 1 μl of Fraction Nuc-III or Cyt-IIIa in 20 μl of 10 mM Tris-HCl, pH 7.6, for 30 min at 37°C, in the presence of indicated concentrations of $MgCl_2$, $CaCl_2$, or $MnCl_2$. Then the DNA was electrophoresed in 0.8% agarose gel. The plasmid DNA incubated in 10 mM Tris-HCl for 30 min at 37°C without adding any nuclease samples was used as a control. Abbreviations used are cir, open circular form; sh, super helical form; lin, linear form. (c) The ends of the DNA fragments extracted from the DEPC-treated nuclei after digestion by Fraction Nuc-III or Cyt-IIIa were examined. The 5′-OH ends and the 3′-OH ends of these DNA fragments were labeled with [γ-^{32}P]ATP and digoxigenin-ddUTP, respectively. DNA samples pretreated with CIP were used as positive controls.

methyl ester, which is an intracellular Ca^{2+}-chelater, promoted their apoptosis [17]. Furthermore, Lazebnik et al. showed that nuclear events of apoptosis could be reproduced in a Ca^{2+} free condition by co-incubating HeLa cell nuclei with extracts from mitotic chicken hepatoma cells [18].

We postulate that the endonuclease activities we semi-purified from P39 cells are possible candidate enzymes involved in apoptosis of myeloid cells. For the delineation of the mechanisms of apoptosis, the relationship between interleukin 1β-converting enzyme related proteases, which are probably crucial in the execution of apoptosis [19-21], and the endonucleases which may be involved in apoptosis would be an important issue for further investigation.

ACKNOWLEDGMENTS

We thank Mr. T. Ohyama for his valuable advice and Ms. Sai Yamada for her excellent technical assistance. This work was supported in part by Grants 03151057, 03671185, 06671088, 07671198, and 08457276 from the Ministry of Education and Grants for Intractable Diseases from the Ministry of Health and Welfare of Japan.

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