Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 247-254

DETECTION OF Mg^{2+}-DEPENDENT ENDONUCLEASE ACTIVITY IN MYELOID LEUKEMIA CELL NUCLEI CAPABLE OF PRODUCING INTERNUCLEOSOMAL DNA CLEAVAGE

Hiroshi Kawabata[1]*, Naoyuki Anzai[1], Hiroshi Masutani[2], Toshiyasu Hirama[2], Yataro Yoshida[2], and Minoru Okuma[2]

[1]First Division, Department of Medicine, Faculty of Medicine,
[2]Institute for Virus Research,
Kyoto University, Sakyo, Kyoto 606, Japan

Received January 19, 1993

SUMMARY: We detected Mg^{2+}-dependent, Ca^{2+}-independent endonuclease activity in non-apoptotic myeloid leukemia cell nuclei using autodigestion method which cleaved the chromatin of the autologous leukemia cells to an oligonucleosomal length pattern. Similar endonuclease activity could be successfully recovered in the protein extracts of the human leukemia cell nuclei. The extracts consistently elicited characteristic DNA cleavage of another leukemia cell (KG-1) nuclei as the target, the enzyme activity of which had been inactivated. We propose that this method is a useful tool for the study of endonucleases involved in apoptosis.

Apoptosis is a common mode of physiological cell death [1] in which an endogenous endonuclease appears to play a major role [2-4]. With some exceptions [5], oligonucleosomal DNA fragmentation detectable as a "ladder pattern" by an electrophoresis on an agarose gel is a useful hallmark of apoptosis [6]. However, the endonuclease(s) responsible for apoptosis has not been isolated to date. Gulcocorticoid-induced apoptosis of thymocyte is accompanied by Ca^{2+}/Mg^{2+}-dependent nuclease synthesis *de novo* [7], but it is not yet clear in other cells whether protein synthesis is necessary to initiate the process, or the nuclease responsible for apoptosis is preexisting.

This study was designed to test the hypothesis that, if all hematopoietic cells can terminate their lives by apoptosis, pertinent endonuclease may preexist. Autodigestion of isolated nuclei is a simple and useful method to examine the endogenous nuclease activity of intact cells [8]. Application of this method enabled us to detect Mg^{2+}-dependent, Ca^{2+}-independent endonuclease activity in myeloid leukemia cell nuclei which cleaved the DNA of their own nuclei to fragments of

*To whom correspondence should be addressed.

Abbreviations: EGTA, ethyleneglycol-bis-tetraacetic acid; DEPC, diethylpyrocarbonate.

0006-291X/93 $4.00

oligonucleosomal lengths. Moreover, we could successfully recover similar enzyme activity from nuclear protein extracts of the myeloid leukemia cells which resulted in characteristic internucleosomal DNA cleavage of isolated nuclei of other target cells, the endogenous enzyme activity of which had been inactivated by treatment with diethylpyrocarbonate (DEPC), a known inactivator of endonucleases [9].

The activity we found may be a potential processor of apoptosis, and the methods we presented here will be applicable to assay apoptosis-associated endonucleases.

MATERIALS AND METHODS

Reagents: RNase A, micrococcal nuclease, DNase I and DNase II were purchased from SIGMA (St. Louis, MO). All other chemicals were obtained from Nacalai Tesque Inc. (Kyoto, Japan) except where otherwise indicated.

Preparation of cell lines: The following cell lines were used. HL60 and KG-1 myelogenous leukemia cells and Raji, B-lymphoblastoid cells were obtained from Japanese Cancer Research Resources Bank, as was P39 myelomonocytoid line originally established from overt leukemia developing from chronic myelomonocytic leukemia [10]. Cells were grown in RPMI 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% heat inactivated fetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA) at 37°C in a humidified 5% CO_2 atmosphere. For all experiments, exponentially growing cells were used.

Preparation of human peripheral lymphocytes: Human peripheral mononuclear cells were isolated from the blood of normal volunteer donors by centrifugation on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient at 400 x g for 30 min. To prepare lymphocytes, the mononuclear cells were suspended in RPMI 1640 supplemented with 10% heat inactivated FBS in a plastic dish, and non-adherent cells were recovered after 2 h incubation at 37°C.

Preparation of rat thymocytes: Male Wistar rats (150-200 g body weight) were anesthetized with ether and promptly sacrificed by cervical dislocation. The thymus was removed and placed in RPMI medium supplemented with 10% heat inactivated FBS. Cell suspensions were prepared by mincing and gently pressing the organ against a fine stainless steel screen followed by pipetting and filtering through a mesh (Falcon Cell Strainer, Becton Dickinson, Lincoln Park, NJ).

Nuclear isolation by hypotonic treatment: To isolate nuclei, 1 x 10^6 - 1 x 10^8 cells were washed with phosphate-buffered saline (PBS, Nikken Bio Medical Laboratory) for 3 times, and were incubated in 50 ml of 1.5 mM $MgCl_2$ for 30 min at 4°C (all subsequent steps were performed at 4°C) on a shaker. The cells were pelleted by centrifugation at 800 x g for 10 min and were gently homogenized in 1.5 mM $MgCl_2$, and the nuclei were collected by recentrifugation (12,000 x g, 10 min). The release of cytoplasm-free nuclei was monitored by phase contrast microscopy.

Autodigestion: The isolated nuclei were incubated in digestion buffer (10 mM Sodium Phosphate pH 7.0, 1 mM $MgCl_2$, unless otherwise indicated) for 16 - 24 h at 37°C. The reaction volume was brought to 100 µl. The reaction was terminated by adding 5 µl of 0.5 M EDTA pH 8.0, 2 µl of 10% sodium dodecyl sulfate and 10 µl of 20 mg/ml proteinase K. Following incubation of the mixture at 50°C for at least 2 hr, the samples were extracted once with phenol/chloroform and once with chloroform and precipitated with 2 volumes of ethanol and 0.1 volume of 3M sodium acetate at -80°C for 20 min. After centrifugation at 14,000 x g for 15 min and rinse with 70% ethanol, the pellet was dissolved in 10 mM Tris-HCl pH 8.0 and 1 mM EDTA containing 1µg/ml of RNase A. The sample was incubated at 37°C for 1 hr, and the DNA concentrations were estimated by measuring the OD_{260}. Two µg/lane of sample DNA was loaded on a 1.5% agarose (FMS Bioproduct, Rockland, ME) gel and electrophoresis was performed in 45 mM of Tris-borate and 1 mM of EDTA (0.5 x TBE buffer) for 12-15 hr. The gel was immersed in 0.5 µg/ml of ethidium bromide for 30 min and photographed under UV light.

Target nuclei digestion: The endonuclease activities of P39 cell nuclear proteins were evaluated by the ability to digest KG-1 cell nuclei as target DNA. The endogenous endonuclease activities of the target KG-1 cell nuclei had been inactivated by pretreatment with DEPC.

Nuclear proteins were extracted by incubating the sample nuclei in 1 ml of 0.6M NaCl for 1 h at 4°C (all subsequent steps were also performed at 0-4°C) on a rotating platform. The nuclear

extracts were centrifuged at 183,000 x g for 1 h to pellet the chromatin materials, and the supernatant was dialyzed against 5 mM Sodium Phosphate pH 7.0. The resulting nuclear protein was re-centrifuged at 14,000 x g for 30 min to remove the trace of chromatin materials. The protein concentration was estimated by measuring the OD_{280} using bovine serum albumin as a standard.

As target substrate, we used KG-1 cell nuclei prepared by hypotonic method as described above. To inactivate the endogenous nucleases, approximately 1 x 10^8 of the nuclei were treated with 10 ml of 0.5% DEPC in 1.5 mM $MgCl_2$ for 1 min at 4°C followed by washing with 1.5 mM $MgCl_2$ for 4 times to remove excess DEPC.

To assay samples for nuclease activity, 1 x 10^6 DEPC-treated target nuclei were incubated with nuclease sample in digestion buffer for 16-24 h at 37°C. The reaction volume was brought to 100 µl. The remaining procedures were the same as autodigestion method.

RESULTS

Endogenous endonuclease of myeloid and lymphoid cells: Using autodigestion method, we consistently found Mg^{2+}-dependent, Ca^{2+}-independent endonuclease activity in the nuclei of myeloid cell lines P39, HL60, and KG-1 which cleaved chromatin of their own nuclei to DNA lengths that were integer multiples of 180-200 bp (Fig.1). The "ladder patterns" were seen in electrophoresis of the DNA derived from nuclei incubated with Mg^{2+} and with Mg^{2+}/EGTA, whereas 2 mM of Ca^{2+} rather diminish the activity. Autodigestion of Raji cells and human peripheral lymphocytes showed a similar electrophoretic pattern (data not shown). We then examined characteristics of the endonuclease activity of P39 cells. Fig.2(a) shows Mg^{2+}-

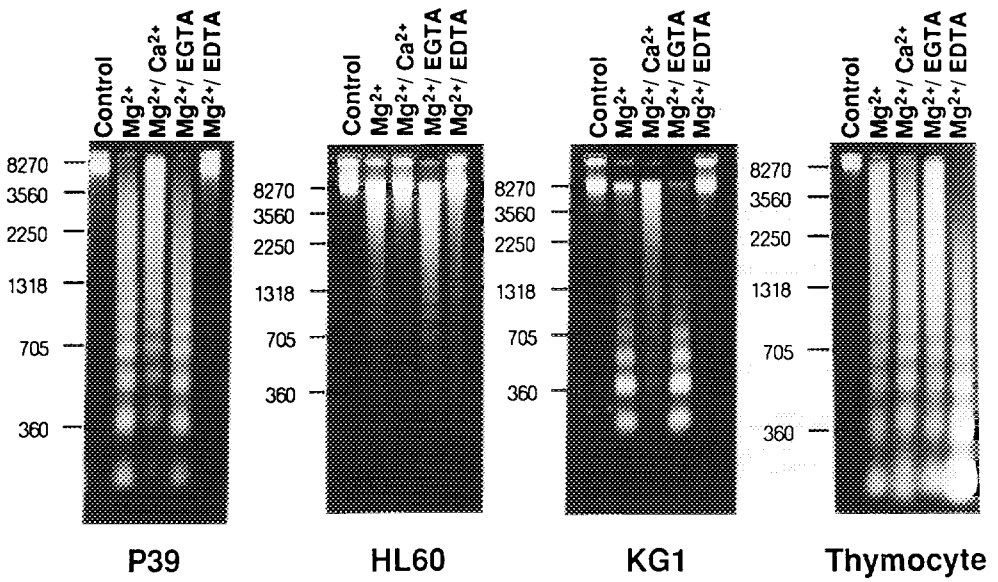

Fig.1. Autodigestions of myeloid leukemia cell and thymocyte nuclei. P39, HL60, KG-1 cell or non-apoptotic rat thymocyte nuclei were incubated in digestion buffer containing 1 mM $MgCl_2$, or 1 mM $MgCl_2$ either with 2 mM $CaCl_2$, 2 mM EGTA or 2 mM EDTA for 24 h at 37°C. DNA extracted from nuclei before incubation was used as controls. DNA molecular weight markers expressed in base pairs are indicated on the left side of these figures.

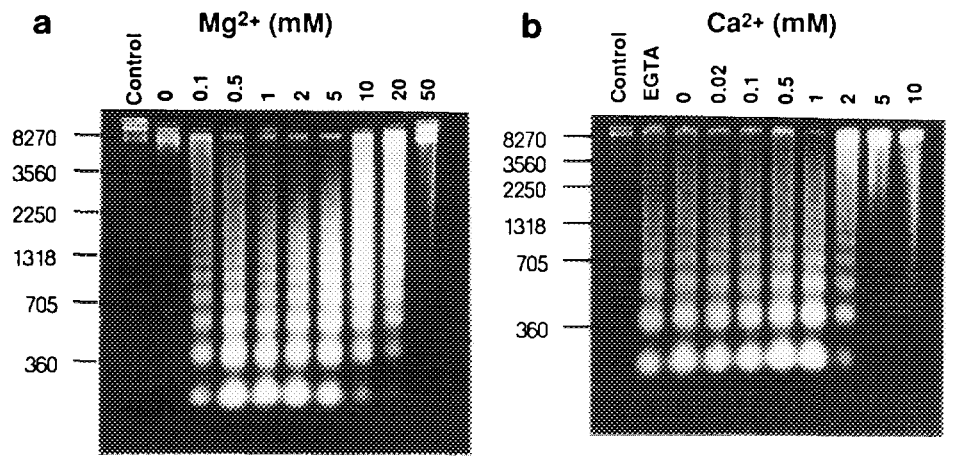

Fig.2. Autodigestion of P39 cell nuclei with various concentrations of Mg^{2+} and Ca^{2+}. DNA extracted from nuclei before incubation was used as controls. (a): P39 cell nuclei were incubated with 0, 0.1, 0.5, 1, 2, 5, 10, 20 and 50 mM $MgCl_2$ for 16 h at 37°C. (b): P39 cell nuclei were incubated with 1 mM EGTA, or 0, 0.02, 0.1, 0.5, 1, 2, 5 and 10 mM $CaCl_2$ in the presence of 1 mM $MgCl_2$ for 16 h at 37°C. DNA molecular weight markers are the same as in Fig.1.

dependent feature of its activity with the optimal Mg^{2+} concentration of 0.5 to 2 mM. In contrast, Fig.2(b) shows that the DNA degrading activity was not affected by either 2 mM of EGTA or 0.02 - 1 mM of Ca^{2+}, suggesting its non-Ca^{2+}-requiring feature. Fig.3 shows the inhibition of the activity by 2 mM of EDTA, 1 mM of Zn^{2+}, 2 mM of ATP, 0.1% of DEPC, and 100 mM of Na^{+}.

Comparison with non-apoptotic thymocytes: In the case of non-apoptotic rat thymocytes, DNA degrading activity was the strongest with 2 mM of EDTA though "DNA ladder" was detectable either with Mg^{2+}-, Ca^{2+}/Mg^{2+}-, or EGTA-containing digestion conditions (Fig.1).

Target nuclei digestion: P39 myeloid cell nuclear proteins extracted by incubation with 0.6M NaCl could elicit internucleosomal DNA cleavage on DEPC treated KG-1 cell nuclei in a dose-dependent manner (Fig.4). The activity did not depend on Ca^{2+}, and, so far as we examined, the features of this activity were identical with those detected by autodigestion system. The nuclear protein lost the activity by heat treatment (70°C, 10 min, data not shown).

Comparison with other nucleases (Fig.5): Micrococcal nuclease and DNase I gave rise to internucleosomal DNA cleavage of DEPC-treated nuclei in the presence of Ca^{2+}. DNase II also induced similar DNA cleavage on DEPC treated nuclei, though the enzyme efficiently cleaved DNA only in the presence of 2 mM EDTA.

DISCUSSION

There are several methods for assaying endonuclease activities [11-13], but most of them have no specificity to nucleases which cleave chromatin at linker regions. In the autodigestion and the

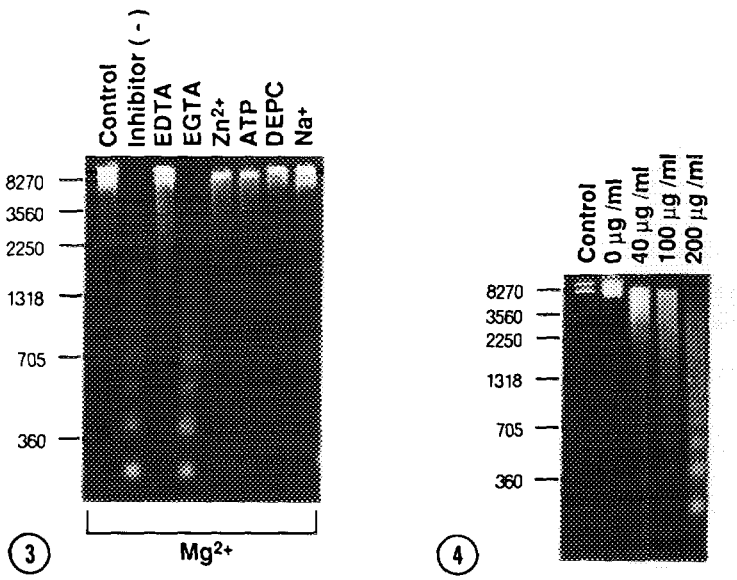

Fig.3. Inhibition of internucleosomal DNA cleavage in autodigestion of P39 cell nuclei. DNA extracted from nuclei before incubation was used as a control. P39 cell nuclei were incubated in digestion buffer containing 1 mM $MgCl_2$ or 1 mM $MgCl_2$ either with 2 mM EGTA, 2 mM EDTA, 1 mM $ZnCl_2$, 2 mM ATP, 0.1% DEPC or 100 mM NaCl for 16 h at 37°C. DNA molecular weight markers are the same as in Fig.1.

Fig.4. Dose-response of endonuclease activity extracted from P39 cell nuclei. DEPC-treated KG-1 cell nuclei were incubated with various amounts(0 - 200µg/ml)of P39 cell nuclear proteins for 16 h at 37°C. DNA extracted from DEPC-treated KG-1 cell nuclei before incubation was used as a control. DNA molecular weight markers are the same as in Fig.1.

target nuclei digestion of our design, we used electrophoretic analysis to detect nuclease activities which predominantly cleave DNA at internucleosomal sites. The advantage of the two methods lies in the fact that both are sensitive measures of nuclease activities contained exclusively within the nuclei free from cytoplasmic interference. Employing these methods, the present study demonstrates the presence in non-apoptotic myeloid cells of Mg^{2+}-dependent but not Ca^{2+} dependent nuclease activity.

Giannakis et.al. have previously suggested that leukemia cells and peripheral lymphocytes contain substantial Mg^{2+}-dependent nuclease activity, but its ability to cleave chromatin at linker regions has not been shown [14]. We have demonstrated typical DNA fragmentation of isolated nuclei during incubation in the presence of Mg^{2+}. Clearly, the DNA fragmentation as detected by the autodigestion is a consequence of Mg^{2+}-dependent, not Ca^{2+}-dependent nuclease activity (Fig.2). We have also demonstrated that similar endonuclease activity in nuclear proteins resulted in internucleosomal DNA cleavage of DEPC-treated target cell nuclei. The only report similar to

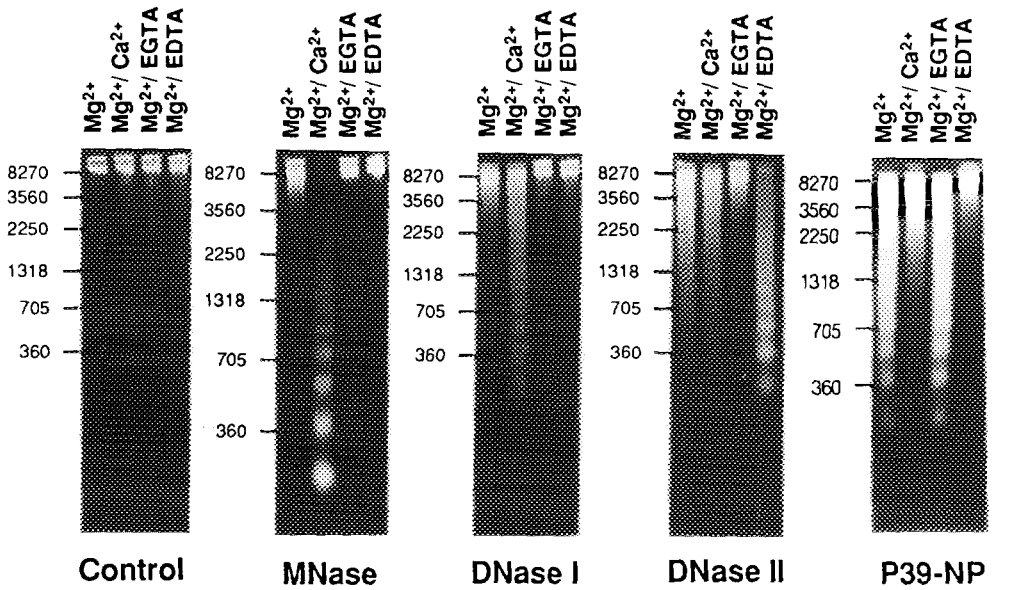

Fig.5. Target nuclei (DEPC-treated KG-1 cell nuclei) digestion by myeloid cell nuclear proteins compared with those by other nucleases. DEPC-treated KG-1 cell nuclei were incubated with micrococcal nuclease (MNase, 100 mU/ml), DNase I (2 μg/ml), DNase II (10 μg/ml) or P39 cell nuclear proteins (P39-NP, 200 μg/ml) for 16 h at 37°C in the presence of 1 mM $MgCl_2$, or 1 mM $MgCl_2$ either with 2 mM $CaCl_2$, 2 mM EGTA or 2 mM EDTA. DEPC-treated nuclei incubated in various digestion conditions without adding any nucleases nor nuclear proteins were used as controls. DNA molecular weight markers are the same as in Fig.1.

our target nuclei digestion is that of Compton, who reported, using nuclear protein extracted from apoptotic chicken thymocytes, "DNA ladder" formation of chicken red blood cell nuclei [15].

Though it is widely held that intracellular Ca^{2+} plays an essential role in apoptosis [16-18], and that Ca^{2+}/Mg^{2+}-dependent nucleases are associated with apoptosis [7, 14, 19], some reports have indicated the presence of apoptosis apparently not requiring calcium ions [20-22]. The endogenous endonuclease activities in myeloid leukemia cell nuclei we detected were not Ca^{2+}-dependent, and the activity was inhibited by Zn^{2+} similar to those shown in previous reports [2, 14, 19,23]. Assuming the activity we detected was identical to one of the nucleases responsible for apoptosis, our results are consistent with Bazar's findings in lymphoid cell system that the DNA fragmentation increases with EGTA and decreases with EDTA [24].

Other well-known nucleases, such as DNase I [25] and DNase II [22], have been suggested as candidates of the nucleases responsible for apoptosis. This prompted us to compare the activity we detected with DNase I or DNase II activities. But previous reports [26,27] and our results shown in Fig.5 demonstrated that the endonuclease activity we detected in myeloid cell nuclei is different

from DNase I or DNase II in its cations requirement. We also compared the endogenous nuclease activities of the myeloid cells with those of rat thymocytes. It is known that the nuclease induced in apoptotic thymocyte is Ca^{2+}/Mg^{2+}-dependent [7], and its activity is enhanced by ATP [15]. but in non-apoptotic thymocyte nuclei, we found a DNase II-like activity acting in the presence of EDTA (Fig.1), which could not be seen in myeloid cell nuclei.

Apoptosis is generally viewed as an active process requiring metabolic energy as well as *de novo* synthesis of RNAs and proteins [1, 7, 28]. But in some systems, inhibition of protein or RNA synthesis can not prevent the DNA degradation but rather induce apoptosis [24, 29, 30]. Conceivably, apoptosis-associated endonucleases constitutively exist in such systems, although they may not readily induce apoptosis without any trigger. Alteration of the relative amounts of nuclear enzymes, or changes of the chromatin structure may be required to give rise to apoptosis. It is possible that the chromatin is decondensed in the hypo-cationic digestion conditions of our system, so that the nuclease may be able to access and cut the linker regions. Similar situations may occur in cells treated with some kinds of apoptosis inducers [20].

In conclusion, we found the Ca^{2+}-independent endonuclease activity in non-apoptotic myeloid leukemia cell nuclei which produce internucleosomal DNA cleavage in autodigestion as well as in target nuclei digestion. We propose that the activity may be one of the apoptosis-associated nucleases. It is hoped that the autodigestion and the target nuclei digestion systems will be useful methods to study apoptosis in a variety of experimental settings.

Acknowledgments: This work was supported in part by Grants (03151057, 03671185) from the Ministry of Education and Grants for Intractable Diseases from the Ministry of Health and Welfare of Japan.

REFERENCES

[1] Wyllie, A. H. (1980) Int. Rev. Cytol. 68, 251-305.
[2] Duke, R.C., Chervenak, R. and Cohen, J.J. (1983) Proc. Natl. Acad. Sci. USA. 80, 6361-6365.
[3] Arends, M. J., Morris, R. G. and Willie, A. H. (1990) Am. J. Pathol. 136, 593-608.
[4] Fesus, L., Davies, P. J. and Piacentini, M. (1991) Eur. J. Cell Biol. 56, 170-177.
[5] Collins, R. J., Harmon, B. V., Gobe, B. V. and Kerr, J. F. R. (1992) Int. J. Radiat. Biol. 61, 451-453.
[6] Wyllie, A. H. (1980) Nature 284, 555-556.
[7] Compton, M. M. and Cidlowski, J. A. (1987) J. Biol. Chem. 262, 8288-8292.
[8] Vanderbilt, J. N., Bloom, K. S. and Anderson, J. N. (1982) J. Biol. Chem. 257, 13009-13017.
[9] Miles, E.W. (1977) Methods Enzymol. 47, 431-442.
[10] Nagai, M., Seki, S., Kitahara, T., Abe, T., Minato, K., Watanabe, S. and Shimoyama, M. (1984) Gann. 75, 1100-1107.

[11] Alexander, M., Heppel, L.A. and Hurwitz, J. (1961) J. Biol. Chem. 236, 3014-3019.
[12] Melgar, E. and Goldthwait, D.A. (1968) J. Biol. Chem. 243, 4401-4408.
[13] Studzinski, G.P. and Fischman, G.J. (1974) Anal. Biochem. 58, 449-458.
[14] Giannakis, C., Forbes, I. J. and Zalewski, P. D. (1991) Biochem. Biophys. Res. Commun. 181, 915-920.
[15] Compton, M. M. (1991) DNA Cell Biol. 10, 133-141.
[16] Bertrand, R., Kerrigan, D., Sarang, M. and Pommier, Y. (1991) Biochem. Pharmacol. 42, 77-85.
[17] Story, M. D., Stephens, L. C., Tomasovic, S. P. and Meyn, R. E. (1992) Int. J. Radiat. Biol. 61, 243-251.
[18] Bellomo, G., Perotti, M., Taddei, F., Mirabelli, F., Finardi, G. and Nicotera, P. (1992) Cancer Res. 52, 1342-1346.
[19] Beletsky, I. P., Matyásová, J., Nikonova, L. V., Skalka, M. and Umansky, S. R. (1989) Gen. Physiol. Biophys. 8, 381-398.
[20] Alnemri, E. S. and Litwack, G. (1990) J. Biol. Chem. 265, 17323-17333.
[21] Lennon, S. V., Kilfeather, S. A., Hallett, M. B., Campbell, A. K. and Cotter, T. G. (1992) Clin. Exp. Immunol. 87, 465-471.
[22] Barry, M. A. and Eastman, A. (1992) Biochem. Biophys. Res. Commun. 186, 782-789.
[23] Cohen, J. J. and Duke, R. C. (1984) J. Immunol. 132, 38-42.
[24] Bazar, L. S. and Deeg, H. J. (1992) Exp. Hematol. 20, 80-86.
[25] Peitsch, M. C., Hesterkamp, T., Polzar, B., Mannherz, H. G. and Tschopp, J. (1992) Biochem. Biophys. Res. Commun. 186, 739-745.
[26] Hewish, D. R. and Burgoyne, L. A. (1973) Biochem. Biophys. Res. Commun. 52, 504-510.
[27] Altenburger, W., Hörz, W. and Zachau, H. G. (1976) Nature 264, 517-522.
[28] Arends, M. J. and Wyllie, A. H. (1991) Int. Rev. Exp. Pathol. 32, 223-254.
[29] Kaufmann, S. H. (1989) Cancer Res. 49, 5870-5878.
[30] Martin, S. J., Lennon, S. V., Bonham, A. M. and Cotter, T. G. (1990) J. Immunol. 145, 1859-1867.