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Formation of 50 kbp chromatin fragments in isolated liver nuclei is mediated by protease and endonuclease activation

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Abstract

Isolated rat liver nuclei were incubated in the presence of divalent cations, and the mechanisms underlying the subsequent chromatin fragmentation were investigated. Either of the two cations, Ca^{2+} or Mg^{2+} was sufficient to produce chromatin fragments with sizes between 700 and 300 kbp. The formation of chromatin fragments of 50 kbp as well as the following internucleosomal DNA cleavage – which are characteristic of apoptosis – were markedly stimulated in the presence of Ca^{2+}. Chromatin degradation to 50 kbp and smaller (oligonucleosome-size) fragments was prevented by inhibitors of endonucleases and serine proteases. We suggest a mechanism whereby the concerted activity of both proteases and endonucleases results in the widespread chromatin cleavage observed in cellls undergoing apoptosis.

Key words: Apoptosis; Chromatin; Endonuclease; Protease; Liver nuclei

1. Introduction

Chromatin condensation in shrinking cells and internucleosomal DNA fragmentation are considered the most characteristic features of apoptosis [1]. While chromatin condensation has long been associated with the occurrence of internucleosomal DNA cleavage by a Ca^{2+}-Mg^{2+}-dependent endonuclease, recent work has suggested that formation of apoptotic bodies precedes DNA laddering [2,3]. These studies have proposed that the formation of HMW DNA fragments with size of 300 kbp and 50 kbp better correlates with the early chromatin alterations visible in pre-apoptotic cells. Internucleosomal cleavage may then be a mid-to late event in apoptosis, mediated by mechanisms probably different from those responsible for the formation of HMW DNA fragments [2,4].

The nature of these large fragments and the mechanism(s) responsible for their formation in apoptotic cells remain unclear. Some studies have concluded that the Ca^{2+}-Mg^{2+}-dependent endonuclease is not involved in the formation of the 50 kbp fragment [3]. Others have suggested that topoisomerase II [2] or protease [4] activation may be required for the formation of the large fragments. Both topoisomerase II-mediated DNA cleavage and proteolysis of chromatin-associated proteins would facilitate the detachment of chromatin loops from nuclear scaffold proteins, reflecting the appearance on agarose gels of fragments with the size of single (50 kbp) or multiple (300 kbp and above) loops.

Recent work in our laboratory has demonstrated that Ca^{2+} is required for the formation of 300 kbp and 50 kbp fragments during the early phases of human thymocyte apoptosis induced by methylprednisolone [5]. In addition, we have shown that the intracellular Ca^{2+} increase elicited by thapsigargin – an inhibitor of the endoplasmic reticular Ca^{2+} pump – is sufficient to cause HMW DNA fragmentation in thymocytes [5]. At variance with other observations [3], these experiments have also shown that endonuclease inhibitors, including Zn^{2+}, can efficiently block the formation of 50 kbp fragments, in methylprednisolone-treated thymocytes [5]. More recent results further support the assumption that intracellular Ca^{2+} accumulation is sufficient to activate HMW DNA fragmentation. Thus, the NMDA/Ca^{2+}-channel blocker, MK-801, prevents the formation of 300 kbp and 50 kbp fragments in cerebellar granule cells exposed to excess glutamate (Ankarcrona, M., Dypbukt, J.M., Zhivotovsky, B., Orrenius, S. and Nicotera, P., unpublished).

To better understand the sequence of events in chromatin cleavage and the role of Ca^{2+} in the formation of HMW DNA fragments, we have designed this study where experiments were carried out in isolated rat liver nuclei. We show that, in the presence of Ca^{2+}, both protease and endonuclease activities are required for the formation of the 50 kbp chromatin fragment, whereas the formation of larger-size fragments has a less specific ion requirement and occurs by distinct mechanisms.

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Abbreviations: ATA, aurintricarboxylic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-*O,O'*-bis(2-aminoethyl-*N,N,N'N'*-tetraacetic acid; HMW DNA, high molecular weight DNA; NMDA, *N*-methyl-D-aspartate; MK-801, (+)-methyl-10-,11-dihydro-5H-benzo(a,d)cyclohepten-5,10-imine hydrogen maleate; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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2. Materials and methods

2.1. Materials

ATA, chromomycin A_3, distamycin A, DTT, N-lauroylsarcosine, spermine, spermidine, TLCK, TPCK were obtained from Sigma. ATP, the inhibitors of calpain I and II, HEPES, PMSF, Proteinase K, RNase A, RNase T_1 were from Boehringer Mannheim. All agarose types were purchased from FMC BioProducts. Inorganic salts were obtained from Riedel-deHaen and Fluka. EDTA, EGTA and sucrose were from Fluka.

2.2. Isolation of nuclei and assay incubation

To remove blood, livers from male, Wistar rats (200–300 g) were cannulated and perfused in situ with an ice-cold TKM solution (70 mM KCl, 50 mM Tris-HCl, pH 7.4), modified by the addition of 0.25 mM spermine, 0.5 mM spermidine, 2 mM EDTA and 0.1 mM PMSF. Livers were then removed and transferred to a TKM solution composed as indicated above but also containing 0.5 mM PMSF, 0.25 M sucrose and 1 mM DTT. They were cut into small pieces and homogenized with a loose fitting pestel (nine strokes). A crude nuclear fraction was isolated by centrifugation at $700 \times g$ for 10 min at 4°C. The pellet was resuspended and homogenized again in the same solution; the homogenate was then filtered through three layers of cheese-cloth and centrifuged at $700 \times g$ for 10 min. The pellet was finally resuspended in the same buffer, homogenized again, and mixed 1:3 (v:v) with cushion buffer (TKM containing 0.5 mM PMSF, 1 mM DTT, 2.3 M sucrose). This mixture was then layered on the cushion and centrifuged at $88,000 \times g$ for 30 min at 4°C. The resulting pellet of highly purified nuclei was resuspended in the incubation medium (120 mM KCl, 2 mM K_2HPO_4, 25 mM HEPES, pH 7.0, 0.3 M sucrose). The nuclear suspension was centrifuged at $600 \times g$ for 5 min at 4°C and finally, the nuclear pellet was resuspended in the incubation buffer. All incubations were performed at 37°C.

2.3. Field-inversion gel electrophoresis (FIGE)

Following treatments, nuclear pellets were centrifuged at $2,000 \times g$ for 40 s and resuspended in a solution containing 0.15 M NaCl, 2 mM KH_2PO_4, pH 6.8, 1 mM EGTA and 5 mM $MgCl_2$. An equal volume of liquified 1% low-melting point agarose solution was added to this suspension, while gently mixing. The mixture was then aliquoted into gel plug casting forms, and allowed to cool and solidify on ice for 10 min. The resulting agarose blocks were transferred into a solution containing 10 mM NaCl, 10 mM Tris-HCl, pH 9.5, 25 mM EDTA, 1% lauroylsarcosine and 200 μg/ml proteinase K, and incubated for 24 h at 50°C with continuous agitation. The plugs were rinsed three times for periods of 2 h at 4°C in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Subsequently, the plugs were stored until use at 4°C in 50 mM EDTA, pH 8.0. FIGE was carried out using a horizontal gel chamber (HE 100B), a power supply (PS 500 XT), and Switchback pulse controller (PC 500) purchased from Hoefer Scientific Instruments (USA). The temperature was controlled by an LKB 2209 Multitemp constant temperature cooling system. Electrophoresis were run at 180 V in 1% agarose gels in 0.5 × TBE (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0), at 12°C, with the ramping rate changing from 0.8 s to 30 s over a 24 h period, applying a forward to reverse ratio of 3:1. DNA size calibration was performed using three sets of pulse markers with overlapping size ranges: chromosomes from *Saccharomyces cerevisiae* (225–2200 kbp), 21 successive concatemers of λDNA (50–1000 kbp), and a mixture of λDNA *Hin*dIII fragments, λDNA and λDNA concatemers (0.1–200 kbp) purchased from Sigma. DNA was stained with ethidium bromide, visualized using a 305 nm UV light source and photographed using Polaroid 665 positive/negative film.

2.4. Conventional agarose gel electrophoresis

Aliquots from the nuclear suspensions were mixed with an equal volume of ice-cold lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100), and incubated at 4°C for 30 min. The resulting suspension was centrifuged at $15,000 \times g$ for 15 min to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernate). The supernates, as well as low-molecular weight DNA released from the agarose plugs during the incubation with proteinase K (see above), were precipitated with a solution of 5 M NaCl in 99% ethanol. The DNA was subsequently loaded on conventional 1.8% agarose gels. Electrophoresis was run with constant current set at 60 mA [1,7]. The mixture of pBR 328 DNA-Bgl 1 and pBR 328 DNA-Hinf 1 was used as a marker. Gel staining and photography were performed as described above.

3. Results

To minimize chromatin damage during isolation of the nuclei, buffers with different ionic strengths were initially tested. When nuclei were isolated in a buffer containing 70 mM KCl, DNA fragmentation, following the isolation procedure, was minimal (Fig. 1, lane 1); fragmentation instead occurred if the KCl concentration in the isolation buffers was decreased. Isolation of the nuclei in presence of 120 mM KCl was also efficient in preventing DNA cleavage, however it also prevented the subsequent fragmentation stimulated by divalent cations (data not shown). These results are consistent with the data published by Filipski et al. [8].

As shown in Fig. 1, incubation of rat liver nuclei in the presence of 10 μM Ca^{2+} and 5 mM Mg^{2+} for 15 min, resulted in the formation of HMW DNA fragments varying in size from 700 kbp to less than 50 kbp (lane 4). In the absence of Mg^{2+}, the addition of 10 μM Ca^{2+} to the incubation buffer was sufficient to produce fragments with sizes of 700 kbp and 300 kbp (lane 3). Millimolar Mg^{2+} concentrations were also able to promote formation of these fragments in the absence of Ca^{2+}, although to a much lesser extent (lane 2). Similar results were obtained when Mg^{2+} was substituted with Mn^{2+}. The formation of 50 kbp fragments was fully prevented when 5 mM EGTA was added to the incubation buffer that included both Ca^{2+} and Mg^{2+}, whereas the formation of 700 kbp and 300 kbp DNA fragments was only delayed. Addition of EGTA also prevented the fragmentation observed when Mg^{2+} was substituted with Mn^{2+} (Fig. 1).

The time dependency of HMW DNA fragmentation in rat liver nuclei is illustrated in Fig. 2A. Large fragments with sizes of 300 kbp and 700 kbp, appeared already after 5 min of incubation of the nuclear fraction in the presence of Ca^{2+} and Mg^{2+}. With time, the fragments larger than 300 kbp disappeared, whereas 50 kbp fragments and, subsequently, oligonucleosome-length fragments accumulated (Fig. 2B). Internucleosomal DNA degradation, as illustrated by the laddering of DNA on agarose gels, appeared 90 min after the beginning of the incubation (Fig. 2B).

To investigate the mechanisms involved in the formation of the HMW DNA fragments, we used a number of agents known to affect the apoptotic process, or to modify chromatin structure. According to their specific targets, these agents can be divided into three groups: inhibitors of proteases, inhibitors of endogenous endonucleases, and agents that can modify chromatin superstructure by binding to specific DNA sites.

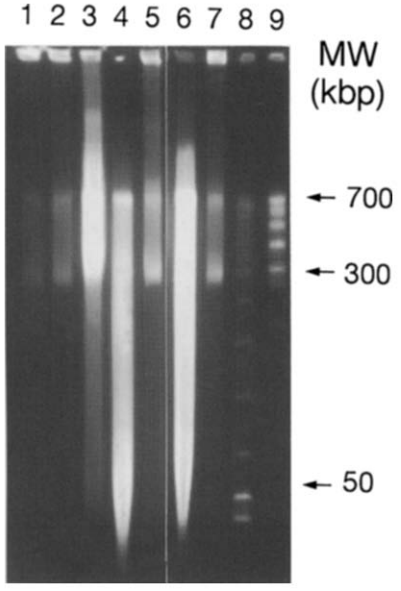

Fig. 1. Effect of divalent cations on the formation of HMW DNA fragments in isolated rat liver nuclei. Nuclei were incubated in the absence (lane 1), or in the presence of 5 mM Mg^{2+} (lane 2), 10 μM Ca^{2+} (lane 3), both Ca^{2+} and Mg^{2+} (lane 4), Ca^{2+}, Mg^{2+} and 5 mM EGTA (lane 5), Ca^{2+} and 2.5 mM Mn^{2+} (lane 6), Ca^{2+}, Mn^{2+} and EGTA (lane 7) for 15 min, at 37°C and examined for HMW DNA fragments by FIGE. Lanes 8 and 9 contained the markers (see section 2).

As shown in Fig. 2A, the chymotrypsin and thiol-protease inhibitor TPCK, and the serine and thiol-protease inhibitor TLCK, blocked the formation of 50 kbp chromatin fragments and delayed the formation of 300 kbp, 700 kbp and even larger fragments. In this case, fragments of very high molecular weight (larger than 1.5 Mbp) distinctly appeared on the gels (lanes 8 and 9). Incubation of isolated nuclei with either of two calpain inhibitors (I and II) reduced the accumulation of 50 kbp fragments, although a sizeable amount of DNA still migrated toward the 50 kbp region (lanes 10 and 11).

Inhibitors of endonucleases such as ATA or Zn^{2+} blocked the formation of the 50 kbp fragments and significantly reduced the accumulation of 300 kbp and 700 kbp fragments (Fig. 2A, lanes 12 and 13). The observation that most of the DNA was retained in the wells suggests in fact, that a considerable portion of the chromatin was still intact (Fig. 2A).

The antibiotic, distamycin A, selectively binds the minor groove of AT-rich DNA [9], and dissociates the histone H1 from an area of contact between DNA loops and the nuclear scaffold proteins, enhancing the cleavage of internucleosomal linkers in these scaffold-associated regions (SARs) [10]. Incubation of isolated nuclei with distamycin A also potentiated the HMW DNA fragmentation induced by divalent cations (Fig. 2A, lane 14). In contrast, chromomycin A_3, which selectively binds the minor groove of GC-rich DNA and does not interfere with histone H1-SARs interactions in vitro [10], blocked the formation of the 50 kbp fragments (Fig. 2A, lane 15).

All of the agents that were most efficient in preventing the formation of the 50 kbp fragments (TPCK, TLCK, ATA, Zn^{2+} and chromomycin A_3) were also able to prevent DNA laddering monitored by conventional gel electrophoresis after 1.5 h incubation (Fig. 3).

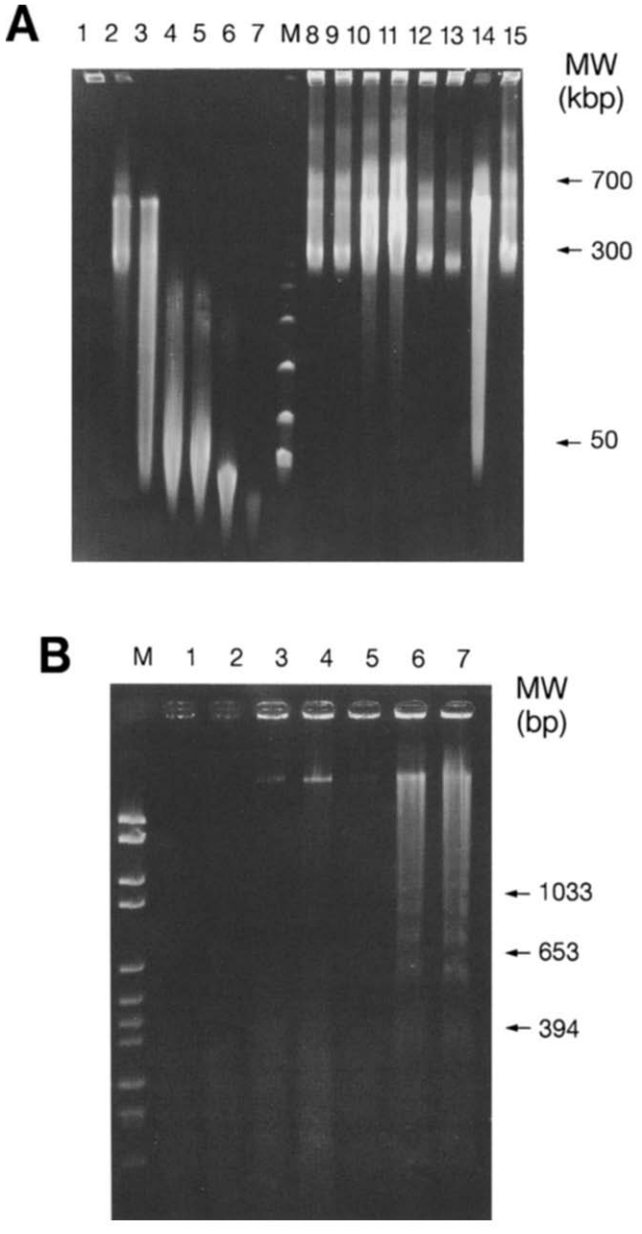

Fig. 2. HMW DNA fragmentation in isolated liver nuclei and its inhibition by various agents. (A) Rat liver nuclei were incubated in the presence of 10 μM Ca^{2+} and 5 mM Mg^{2+} for: 0, 5, 15, 30, 60, 90 and 120 min (lanes 1–7) or for 30 min in the presence of Ca^{2+}, Mg^{2+} and: 10 μM TPCK (lane 8), 50 μM TLCK (lane 9), 20 μM calpain inhibitor I (lane 10) or 20 μM calpain inhibitor II (lane 11), 0.3 mM ATA (lane 12), 100 μM Zn^{2+} (lane 13), 2.5 μM distamycin A (lane 14), and 5 μM chromomycin A_3 (lane 15). M = marker. (B) DNA that leaked from the agarose plugs was analyzed by conventional gel electrophoresis. Lanes 1–7 corresponds to lanes 1–7 in A. M = marker.

Discussion

Substantial evidence indicates that sustained intracellular Ca^{2+} elevations are sufficient to promote all the characteristic changes of apoptosis including chromatin condensation, apoptotic body formation, HMW DNA fragmentation and DNA laddering in several experimental systems [5,7,11–13]. In particular, recent studies in human thymocytes have revealed that Ca^{2+}-dependent endonuclease activity is required for the formation of 50 kbp DNA fragments in glucocorticoid-induced apoptosis [5]. Our present results show that either of the two cations, Ca^{2+} or Mg^{2+}, is sufficient to promote the formation of the large chromatin fragments from 700 to 300 kbp in isolated liver nuclei. However, the 50 kbp band appeared in a relatively short time (15 min) only when both cations were included in the nuclear incubation. Thus, the single addition of either Ca^{2+} or Mg^{2+} eventually resulted in the formation of the 50 kbp fragment, after a much longer time (i.e. over 2 h; data not shown). Notably, micromolar Ca^{2+} levels were as efficient as millimolar Mg^{2+} concentrations to promote the appearance of the 700 kbp and the 300 kbp bands. Since Mg^{2+} was effectively substituted by Mn^{2+}, and Ca^{2+} chelation by EGTA did not block the accumulation 700 kbp and 300 kbp fragments, it appears that the cation requirement for the formation of fragments up to 300 kbp was not selective. Thus, should these findings apply to the situation in apoptotic cells, only the subsequent chromatin cleavage (i.e. the formation of 50 kbp fragments) would be critical for cell death and involve the activation of selective Ca^{2+}-dependent mechanisms. This assumption is supported by the following considerations: (i) a variety of agents that protect from apoptotic cell killing prevent the generation of 50 kbp chromatin fragments but not the formation of those with a size of 700 and 300 kbp; (ii) more specifically, Ca^{2+} chelators and channel blockers prevent the formation of the 50 kbp band and the killing of either glucocorticoid-treated thymocytes [5] or glutamate-treated neurons (Ankarcrona, M., Dypbukt, J.M., Zhivotovsky, B., Orrenius, S. and Nicotera, P., unpublished). Notably, chromatin digestion in cells undergoing apoptosis produces fragments of sizes identical to those found after chromatin digestion in isolated liver nuclei, suggesting that the pattern of DNA cleavage reflects a periodicity in the chromatin structure.

These results also show that chromatin cleavage involves at least two distinct steps: the formation of fragments with sizes of 300 kbp and larger and the subsequent generation of 50 kbp fragments followed by internucleosomal cleavage. While the first step proceeds without specific requirement for different cations the second step of chromatin cleavage (degradation to 50 kbp fragments) involves the activation of Ca^{2+}-dependent enzymes. Confocal microscopy imaging has suggested that the formation of the 50 kbp fragments is associated with

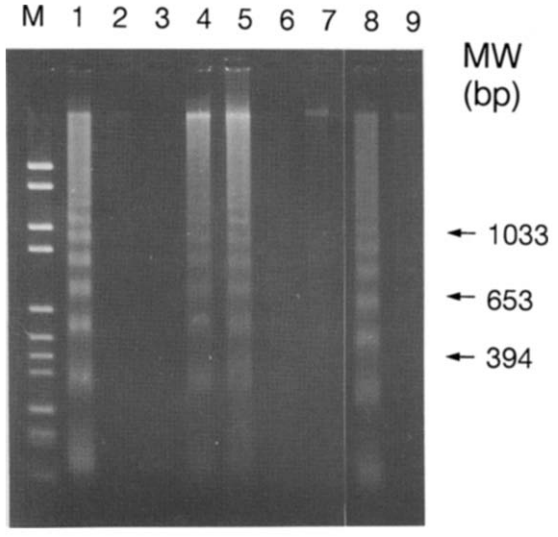

Fig. 3. DNA laddering in isolated liver nuclei and its inhibition by various agents. Rat liver nuclei were incubated in the presence of 10 μM Ca^{2+} and 5 mM Mg^{2+} for 90 min alone (lane 1) or in the presence of Ca^{2+}, Mg^{2+} and 10 μM TPCK (lane 2), 50 μM TLCK (lane 3), 20 μM calpain inhibitor I (lane 4) or 20 μM calpain inhibitor II (lane 5), 0.3 mM ATA (line 6), 100 μM Zn^{2+} (lane 7), 2.5 μM distamycin A (lane 8), and 5 μM chromomycin A_3 (lane 9). M = marker.

major rearrangements in chromatin structure [14]. Further, recent studies in thymocytes have shown that the accumulation of 50 kbp fragments seems to be a critical step for the formation of apoptotic bodies (Zhivotovsky, B., Wade, D., Nicotera, P. and Orrenius, S., unpublished). Our present results show that both protease and endonuclease activities play a prominent role in the formation of these chromatin fragments.

A critical role for proteases in cell death has been considered by several laboratories [15–17]. Thus, serine proteases may be involved in CTL- and in TNFα-induced apoptosis [15,16]. In the present work, inhibitors of serine proteases TPCK and TLCK, prevented the formation of 50 kbp fragments (see Fig. 2A) as well as the subsequent internucleosomal DNA fragmentation (see Fig. 3). Calpain inhibitors also decreased the formation of 50 kbp fragments, although they were not equally effective as TPCK and TLCK. Notably, the calpain inhibitors were also not very effective in preventing subsequent internucleosomal DNA fragmentation (see Fig. 3). However, it should be noted that there is a large variability in permeability properties and specificity of these agents, that makes the comparison of their relative potency rather difficult. The findings that nuclease inhibitors such as ATA and Zn^{2+} also prevented the formation of 50 kbp fragments and the subsequent internucleosomal cleavage support a role for endonucleases at this stage.

Thus it may be concluded that, in the presence of calcium ions, both protease and endonuclease activities intervene to promote widespread chromatin cleavage. At this point, one may only speculate that cleavage of ma-

trix proteins is required to increase DNA accessibility to the endonuclease. Notably, very recent work has shown that the association of a Ca^{2+}-regulated serine protease is involved in lamin proteolysis with the nuclear scaffold [18]. Lamins are intrinsically connected and linked to chromatin loop domains by means of DNA–protein interactions and by scaffold-associated proteins [19]. Therefore, lamin dissociation or loss of contact between chromatin loops and nuclear scaffold elements may result in detachment of large chromatin regions. It has been shown that selective titration of AT-rich tracts by distamycin A abolishes the interaction of histone H1 with SARs and specifically enhances cleavage of internucleosomal linkers of SARs [10]. In our experiments distamycin A also very efficiently enhanced the chromatin cleavage in presence of divalent cations. In contrast, chromomycin A_3, which does not interfere with SAR–protein interactions in vitro [10], did not enhance the early steps of chromatin cleavage and prevented formation of 50 kbp fragments. It seems therefore likely that either lamin proteolysis or loop detachment from scaffold elements may initiate the irreversible chromatin fragmentation in this system. Exposure of chromatin to cleavage by endonucleases may thereby occur and lead to complete digestion of the genome to its minimum-sized constituents.

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