

# DNA fragmentation into 200–250 and/or 30–50 kilobase pair fragments in rat liver nuclei is stimulated by $Mg^{2+}$ alone and $Ca^{2+}/Mg^{2+}$ but not by $Ca^{2+}$ alone

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## Abstract

Internucleosomal cleavage of DNA has often been regarded as the biochemical hallmark of apoptosis. We now demonstrate in isolated rat liver nuclei that DNA is initially cleaved into  $\geq 700$ , 200–250 kbp and 30–50 kbp fragments via a multi-step process, which is activated by  $Mg^{2+}$  and  $Mg^{2+}+Ca^{2+}$  but not by  $Ca^{2+}$  alone. The subsequent internucleosomal cleavage requires both cations. These findings demonstrate that a key event in the apoptotic process is the fragmentation of DNA into large kbp fragments by either a  $Mg^{2+}$ -dependent process (which can be potentiated by  $Ca^{2+}$ ) and/or by a  $Ca^{2+}/Mg^{2+}$  activated endonuclease(s).

**Key words:** Apoptosis; DNA fragmentation; Magnesium; Endonuclease

## 1. Introduction

Two classical morphological features of apoptosis are the condensation of chromatin and its margination at the nuclear boundary [1]. Internucleosomal fragmentation of DNA, which is seen as a 'ladder' when the DNA is subjected to conventional agarose gel electrophoresis [2], has been regarded as the biochemical hallmark of apoptosis in many different cell types (see [3,4] for reviews). Internucleosomal cleavage of DNA is believed to be due to the action of one or possibly more  $Ca^{2+}/Mg^{2+}$ -dependent endonucleases [3]. Some studies have suggested that increases in cytosolic  $Ca^{2+}$  concentrations precede apoptotic cell death. Thus, for example, Wyllie et al. [5] showed that the calcium ionophore A23187 activated DNA cleavage and apoptosis in thymocytes whilst McConkey et al. [6] demonstrated that glucocorticoids induce the endonuclease activity via an elevation of cytosolic  $Ca^{2+}$  levels. Chelation of intracellular  $Ca^{2+}$  with quin-2-AM was found to prevent both the activation of the endonuclease and the onset of apoptosis [6–8]. Further evidence for the role of  $Ca^{2+}$  in endonuclease mediated fragmentation of DNA has come from studies on isolated rat liver nuclei, which have shown that  $Ca^{2+}$  activates an endonuclease producing a fragmentation pattern identical to that seen in apoptotic cells [9].

However, recent studies [10,11] have demonstrated that DNA cleavage in apoptotic thymocytes involves an initial cleavage into high molecular weight fragments of approximately  $\geq 700$ , 200–250 and 30–50 kbp, followed by subsequent endonuclease cleavage into nucleosome or oligonucleosome sized fragments. It was proposed [10]

that the formation of large fragments of DNA represents a much earlier event in the apoptotic process. In support of this conclusion, it has been shown that isolated hepatocytes undergoing apoptosis with TGF- $\beta_1$  treatment exhibit the classical morphology of condensed chromatin but without DNA laddering [12]. Furthermore, recent studies by Oberhammer et al. [13] have shown that in a variety of cell lines, apoptotic cell death occurs with cleavage of the DNA into 300 and/or 50 kb fragments prior to, or in the absence of internucleosomal fragmentation.

These findings suggest that the degradation of DNA into large kbp sized fragments is a key event in the apoptotic process and it is important to characterise the mechanisms responsible for this phenomenon. In the present study, we have developed an *in situ* end labelling (ISEL) technique, which in conjunction with flow cytometry can be used to detect DNA strand breaks in isolated nuclei.

This method has been used with conventional agarose gel and field inversion gel electrophoresis (FIGE) to show that the initial cleavage of DNA in isolated rat liver nuclei into 200–250 and/or 30–50 kbp fragments is a  $Mg^{2+}$  and/or  $Mg^{2+}+Ca^{2+}$ -dependent process. The subsequent internucleosomal cleavage has an absolute requirement for both  $Mg^{2+}$  and  $Ca^{2+}$ . The results support the hypothesis that the large fragment formation and internucleosomal cleavage are two distinct processes, which are involved in DNA degradation during apoptosis.

## 2. Materials and methods

### 2.1. Chemicals

Terminal deoxynucleotidyl transferase (TdT) was from Gibco, Paisley, Scotland, UK and digoxigenin-11-2'-deoxy-uridine-5'-triphos-

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phate (Dig-11-dUTP) and antidigoxigenin-fluorescein, Fab fragments (anti-Dig) were obtained from Boehringer-Mannheim UK, Bell Lane, Lewes, East Sussex, UK. Other chemicals were obtained from Sigma Chemical Co., Poole, Dorset, UK.

## 2.2. Preparation of rat liver nuclei in magnesium buffers (Mg-buffers) and spermidine/spermine containing buffers (SP-buffers)

Rat liver nuclei were prepared as described by Jones et al. [9] but with some modifications. Briefly, male F344 rats (180–200 g; Harlan-Olac, Bicester, Oxfordshire, UK) were anaesthetised i.p. with 0.6 ml of 60 mg/ml sodium pentobarbitone in isotonic saline and the liver perfused *in situ* with ice-cold TKM buffer (50 mM Tris-HCl, 25 mM KCl, 5 mM  $MgCl_2$ , pH 7.5). The liver was removed and homogenised in 40 ml of S-TKM (TKM + 0.25 M sucrose) and the homogenate filtered through nylon bolting cloth (200  $\mu$ m and 70  $\mu$ m mesh size). The homogenate was centrifuged for 10 min (700  $\times$  g) in a Beckman JS-13.1 rotor and the pellet resuspended in 40 ml of S-TKM which was centrifuged as before. The pellet from this spin was resuspended in S-TKM (24 ml) and mixed with 48 ml of S1-TKM (TKM + 2.3 M sucrose) before aliquoting into four centrifuge tubes, each containing a 6 ml 'cushion' of S1-TKM. The tubes were centrifuged for 47,000  $\times$  g/1 h in a Beckman SW 28 Ti rotor and afterwards the upper layer and sucrose 'cushion' discarded before gently resuspending the pellet in S1-TKM (10 ml) and centrifuging for 1000  $\times$  g/5 min. The final pellet was resuspended in 10 ml of incubation buffer (125 mM KCl, 2 mM potassium phosphate, 25 mM HEPES, 4 mM  $MgCl_2$ , pH 7.0).

Rat liver nuclei were also prepared in the above buffers (SP-buffers) in which the  $Mg^{2+}$  was replaced with 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA and 0.1 mM EGTA. The incubation/final resuspension buffer contained 0.15 mM spermine and 0.5 mM spermidine but no EDTA or EGTA. Nuclei were counted using a Coulter-ZM cell counter and 256 Channelyzer (Coulter Electronics, Luton, Beds., UK). Lower and upper thresholds were set to count between 3.52  $\mu$ m and 9.25  $\mu$ m for unfixed nuclei, and 2.8 mm and 7.34 mm for fixed nuclei which were smaller in size. Representative samples of the nuclei preparations were examined by light and electron microscopy and were found to be intact and free of contamination from other subcellular organelles.

## 2.3. Autodigestion and electrophoresis of DNA

Nuclei ( $10 \times 10^6$ /ml) were incubated in the appropriate buffer with and without additions at 37°C for 30 min. The reaction was stopped with an equal volume of ice-cold incubation buffer containing 20 mM EGTA and aliquots taken for conventional agarose gel electrophoresis which was carried out as described previously [14] using  $5 \times 10^5$  nuclei. Agarose plugs containing  $5 \times 10^5$  nuclei were prepared by the method of Anand and Southern [15] and digested with 1 mg/ml Pronase for 48 h at 50°C before storing at 4°C prior to field inversion gel electrophoresis (FIGE), carried out as described by Brown et al. [10].

Preliminary experiments (results not shown) with Mg-buffers showed that DNA ladders could be induced with as little as 10  $\mu$ M  $Ca^{2+}$ . These studies also demonstrated that nuclei prepared and incubated in the standard Mg-buffers without calcium at 37°C for 30 min showed a significant amount of endogenous DNA laddering. However,  $Mg^{2+}$  which is normally used to stabilise the DNA, can be replaced with spermine and/or spermidine [16,17] and this in conjunction with EDTA and EGTA to remove potential endonuclease and endoprotease activating cations (e.g.  $Ca^{2+}$ ; personal communication, L. Kokileva) helps to preserve the DNA structure. The nuclei prepared/incubated in SP-buffers did not show endogenous laddering even when incubated for 4 h at 37°C (Fig. 5) and the level of ISEL was the same as in freshly isolated nuclei (e.g. Figs. 3,4). Therefore, all subsequent experiments were carried out in the SP-buffers.

## 2.4. *In situ* end labelling (ISEL) flow cytometric analysis of DNA

ISEL on isolated nuclei was carried out using a modified procedure of the method described by Gold et al. [18]. Nuclei ( $20 \times 10^6$ /2 ml) autodigestion was carried out as described above and the reaction stopped with 2 ml of ice-cold buffer (+ 20 mM EGTA). The nuclei were sedimented (1000  $\times$  g/5 min) and resuspended in PBS (100  $\mu$ l) before fixing for 15 min at 4°C in 1 ml of 1% formaldehyde, Hanks buffered salt solution, 20 mM HEPES, pH 7.0. The nuclei were pelleted/resuspended in PBS (100  $\mu$ l) before adding 2 ml of 70% EtOH (–20°C) and permeabilising for 1 h at 4°C. Sedimented nuclei were resuspended in

1 ml of TBS (50 mM Tris, 150 mM NaCl, pH 7.6 buffer) before Coulter counting. The nuclei were pelleted and resuspended to give  $2 \times 10^6$  nuclei/100  $\mu$ l in TdT labelling buffer (100 mM sodium cacodylate, 10 mM  $CoCl_2$ , 1 mM DTT, pH 7.2). Nuclei (0.1 ml) were then incubated with TdT enzyme (15 units) and Dig-11-dUTP (final concentration 2  $\mu$ M) for 30 min/37°C before terminating the reaction with ice-cold TBS+5 mM EDTA (1 ml). Nuclei were resuspended in 0.5 ml of antibody labelling buffer (5% non-fat dried milk, 0.1% (v/v) Triton X-100, 4  $\times$  standard saline citrate, pH 7.2) and incubated for 15 min/4°C to block non-specific antibody binding before centrifuging at 1500  $\times$  g/5 min. The pellet was resuspended in 0.25 ml of antibody buffer containing a 1/125 dilution of anti-Dig antibody and incubated in the dark for 30 min at 37°C before sedimenting and resuspending the nuclei (1 ml) in PBS containing 10  $\mu$ g/ml propidium iodide.

A FACScan flow cytometer (Becton Dickinson, USA) was used to measure the red (PI) and green (fluorescein; anti-Dig) fluorescence of labelled nuclei. Using doublet discrimination, data from  $10^4$  singlet nuclei were collected and displayed as bivariate cytograms of red versus green fluorescence. Typically, this gave two populations (see Fig. 3) which from the PI fluorescence corresponded to diploid (2N) and tetraploid (4N) nuclei. LYSYS II software was used to gate around these populations to measure the mean relative green fluorescence intensity (designated peak mean fluorescence) of the fluorescein labelled nuclei and thereby the increase in DNA strand breakage.

## 3. Results

### 3.1. $Mg^{2+}$ induced DNA fragmentation in rat liver nuclei

Several studies have shown that  $Ca^{2+}$  stimulates endonuclease activity in isolated nuclei (see Schwartzman and Cidlowski [4], for review). In the case of thymocyte nuclei, relatively large, non-physiological concentrations of  $Ca^{2+}$  (1–5 mM) have been used to induce DNA laddering [19], whereas in rat liver nuclei, micromolar concentrations have been reported to be effective [9]. When nuclei were incubated in SP-buffers for 30 min/37°C and without added  $Ca^{2+}$  or  $Mg^{2+}$  there was no endogenous DNA cleavage as shown by agarose gel electrophoresis and FIGE (Fig. 1, lanes 1–2). However, the addition of 4 mM  $Mg^{2+}$  alone (Fig. 1, lanes 10–11) resulted in the formation of large fragments of DNA with an approximate size of 200–250 kbp and without any detectable DNA laddering. Calcium on its own did not induce large fragment formation or internucleosomal cleavage (Fig. 1, lanes 12–13).

### 3.2. $Mg^{2+}$ induced DNA fragmentation is facilitated by $Ca^{2+}$

When  $Ca^{2+}$  was added with 4 mM  $Mg^{2+}$ , a more extensive cleavage of DNA was observed and the size of the DNA fragments produced was markedly dependent on the concentration of  $Ca^{2+}$  (Fig. 1, lanes 3–9). At 10 and 25  $\mu$ M  $Ca^{2+}$  (lanes 3,4) there was, initially, formation of 200–250 and 30–50 kbp fragments without any internucleosomal cleavage. Between 50 and 100  $\mu$ M  $Ca^{2+}$  (lanes 5–6), DNA cleavage was progressed further and predominantly 30–50 kbp fragments were detected with a small amount of laddering. Above 100  $\mu$ M  $Ca^{2+}$ , smaller amounts of large fragments were detected, accompanied by a progressive increase in internucleosomal cleavage until ultimately only the classical DNA ladder (Fig. 1,

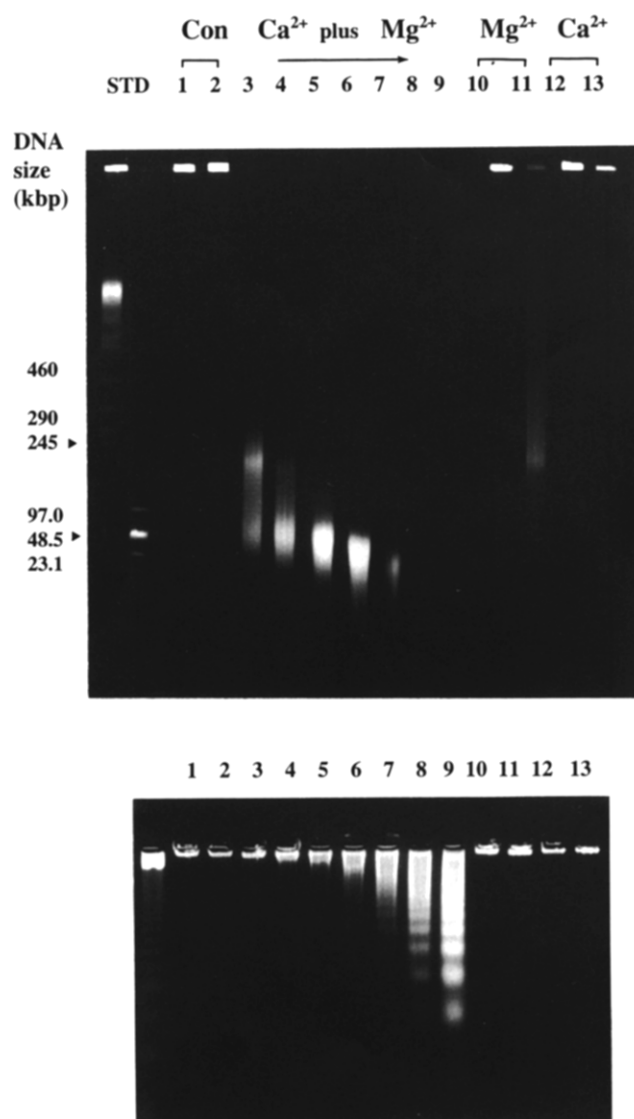


Fig. 1. Conventional agarose gel and field inversion gel electrophoresis (FIGE) of rat liver nuclei after  $Mg^{2+}$  and  $Mg^{2+}$  plus  $Ca^{2+}$  activation. Rat liver nuclei were isolated and incubated in SP-buffers and subsequently analysed by FIGE (upper panel) and agarose gel electrophoresis (lower panel) as described in section 2. In the FIGE gel the first two lanes contain *S. cerevisiae* chromosome standards (243–2200) and (0.1–200) kbp standards, respectively, and key sizes are indicated (▶). The remaining 13 lanes are as follows: con (1 & 2) = 0 min and 30 min control without  $Ca^{2+}$  and  $Mg^{2+}$ ;  $Ca^{2+}$  plus  $Mg^{2+}$  (3–9) = 10, 25, 50, 100, 200, 400, 800  $\mu M$   $Ca^{2+}$  respectively with constant 4 mM  $Mg^{2+}$ ;  $Mg^{2+}$  (10 & 11) = 0 and 30 min with 4 mM  $Mg^{2+}$ ;  $Ca^{2+}$  (12 & 13) = 0 and 30 min with 200  $\mu M$   $Ca^{2+}$ . In the agarose gel the first lane contains markers of 123 bp or multiples thereof. The remaining lanes are as for the FIGE gel.

lanes 7–9) was detected. This data demonstrates that  $Ca^{2+}$  facilitates or potentiates the  $Mg^{2+}$ -dependent DNA degradation.

### 3.3. Size distribution of DNA fragments after $Mg^{2+}$ induced DNA degradation

Varying the  $Mg^{2+}$  concentration significantly affected the size distribution of kbp sized DNA fragments

(Fig. 2). Thus at 1 mM  $Mg^{2+}$  there was no DNA fragmentation (lane 3, Fig. 2). With 2 mM (lane 4) the DNA was degraded into very large fragments, i.e. mainly  $\geq 700$  kbp and at 4 mM  $Mg^{2+}$  (lane 5), both  $\geq 700$  kbp and 200–250 kbp fragments were produced. The latter size predominated after incubation with 8 mM  $Mg^{2+}$  (lane 6), although there was evidence that some of the DNA was further degraded into the smaller 30–50 kbp fragments. When the  $Mg^{2+}$  concentration was increased to 16 mM (lane 7), the major DNA species was the 200–250 kbp fragments suggesting that the increased concentration of

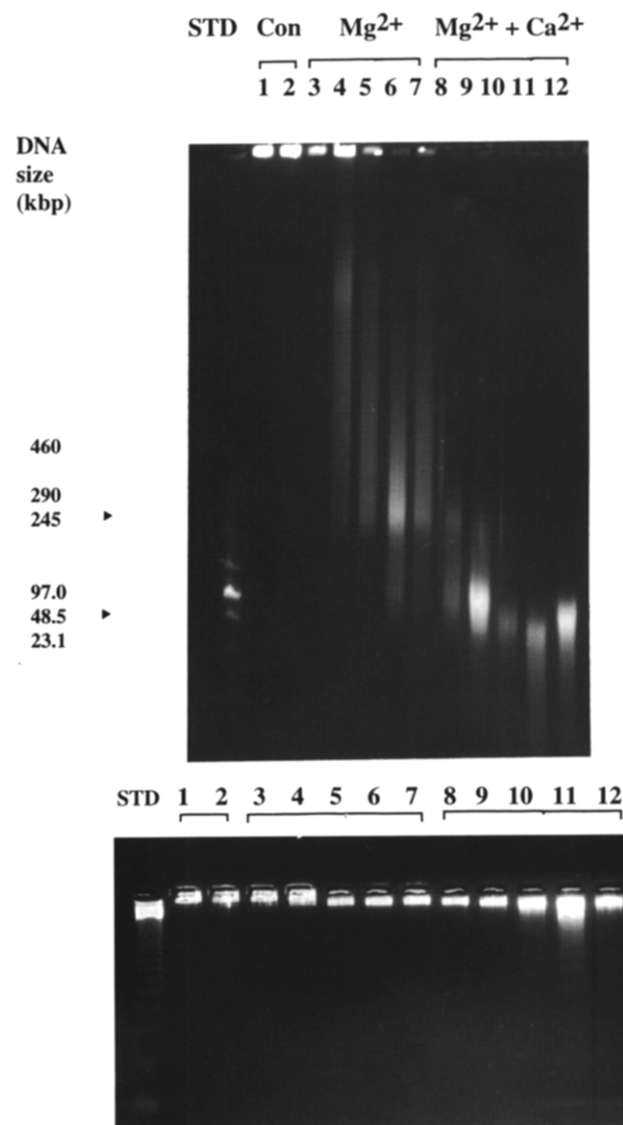


Fig. 2. Conventional agarose gel and field inversion gel electrophoresis (FIGE) of  $Mg^{2+}$  induced DNA fragmentation in rat liver nuclei. Rat liver nuclei prepared as described in Fig. 1 were analysed by FIGE (upper panel) and agarose gel electrophoresis (lower panel) as described above. In the FIGE gel first two lanes contain standards as described in Fig. 1 and key sizes are indicated with arrows. Lanes 1 & 2 are the 0 and 30 min controls respectively; lanes 3–7 are 1, 2, 4, 8 and 16 mM  $Mg^{2+}$  minus calcium; lanes 8–12 are 1, 2, 4, 8 and 16 mM  $Mg^{2+}$  plus 200  $\mu M$   $Ca^{2+}$ . In the agarose gel the first lane is the 123 bp standards and lanes 1–12 are as described for FIGE.

magnesium was inhibiting the formation of the 30–50 kbp fragments.

When the  $Mg^{2+}$  concentration was varied in the presence of a fixed concentration of  $Ca^{2+}$  (200 mM), a markedly different profile of DNA large fragments was observed (Fig. 2, lane 8–12). Thus, at 1 mM  $Mg^{2+}$  (lane 8) the 30–50 kbp fragment predominated with only a small amount of 200–250 kbp sized DNA being detected. With 2 mM  $Mg^{2+}$  (lane 9), only the 30–50 kbp fragment was detected. At 4 and 8 mM  $Mg^{2+}$  (lanes 10,11) the major fragments were <30 kbp and this was accompanied as shown in the lower panel of Fig. 2 with a small amount of DNA laddering with 4 mM  $Mg^{2+}$  (lane 10) and more extensive laddering with 8 mM  $Mg^{2+}$  (lane 11). At 16 mM  $Mg^{2+}$  (lane 12) there was a small amount of DNA laddering, suggesting that internucleosomal cleavage was inhibited at high  $Mg^{2+}$  concentrations. Thus in the presence of  $Mg^{2+}$  and  $Ca^{2+}$  a more extensive cleavage of DNA was observed than in the presence of  $Mg^{2+}$  alone.

### 3.4. *In situ* end labelling (ISEL) and flow cytometry as a semi-quantitative measure of DNA strand breakage

Although the gel electrophoresis experiments gave valuable information on the size of the DNA fragments, it

was difficult to quantify the extent or the rate of the DNA cleavage. Therefore, we used ISEL to measure the amount of DNA strand breakage in isolated rat liver nuclei and thereby quantitate the effects of  $Mg^{2+}$  and  $Ca^{2+}$  on DNA degradation. A typical cytogram is shown in Fig. 3 in which the data are displayed as 3D contour plots with DNA content (PI, red fluorescence) in the *x*-axis and Anti-Dig labelling (green fluorescence) which is a measure of DNA cleavage shown in the *z*-axis. The cytogram demonstrates that this technique gives information about both 2*N* and 4*N* nuclei populations. The level of ISEL for nuclei incubated for 30 min (without any divalent cations) at 37°C was very low (Fig. 3A) and was equivalent to non-incubated nuclei (data not shown). However, when 8 mM  $Mg^{2+}$  was added there was a small but measurable increase in the ISEL (Fig. 3B). In contrast the addition of 200 mM (Fig. 3C) and 800 mM (data not shown).  $Ca^{2+}$  alone did not produce any increase in the level of ISEL. However, the addition of  $Ca^{2+}$  (200  $\mu$ M) plus  $Mg^{2+}$  produced the greatest increase in ISEL (Fig. 3D). In all cases the level of ISEL in the two populations of nuclei was related to the DNA content, i.e. end labelling in tetraploid nuclei was approximately double that of the diploid nuclei. Thus, the ploidy

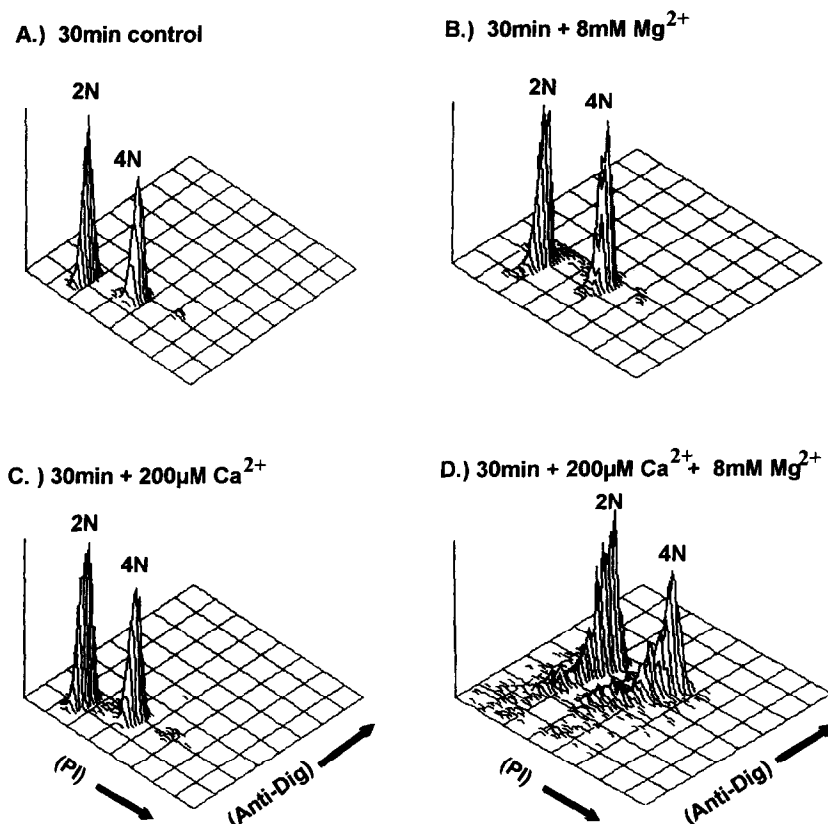


Fig. 3. Flow cytometric detection of DNA fragmentation in isolated rat liver nuclei using *in situ* end labelling (ISEL). Rat liver nuclei were isolated in SP-buffers as described in section 2 and then incubated as indicated, i.e. (A) 30 min control with no  $Mg^{2+}$  or  $Ca^{2+}$  at 37°C, (B) as (A) but with the addition of 8 mM  $Mg^{2+}$ , (C) as (A) but with the addition of 200  $\mu$ M  $Ca^{2+}$ ; (D) as (A) but with the addition of 200  $\mu$ M  $Ca^{2+}$  and 8 mM  $Mg^{2+}$ . The reactions were then terminated and the DNA strand breakage determined by ISEL and flow cytometry as described in section 2. The figure shows the data represented as a 3D cytogram with relative PI fluorescence (DNA content) and green fluorescence (anti-DIG) plotted as indicated.

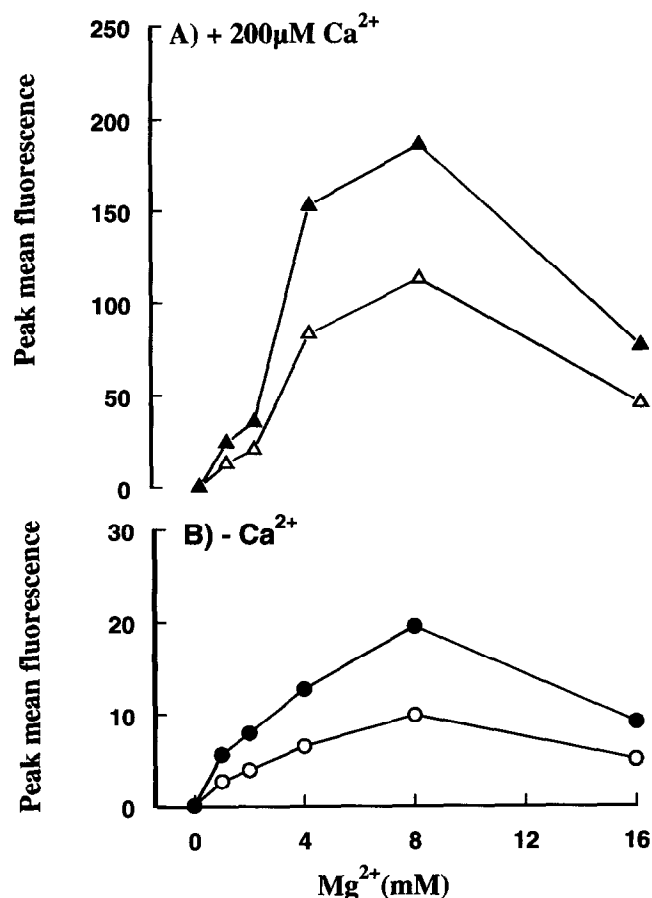


Fig. 4. Effect of Ca<sup>2+</sup> on Mg<sup>2+</sup> induced ISEL cleavage in rat liver nuclei. Rat liver nuclei were prepared as described in Fig. 1 and then incubated for 30 min at 37°C in the SP-buffer supplemented with (A) and without 200 μM Ca<sup>2+</sup> (B), and containing increasing concentrations of Mg<sup>2+</sup>. DNA cleavage was measured with ISEL and flow cytometry as described in section 2. Open and solid symbols represent 2N and 4N nuclei, respectively.

of the nuclei did not alter the susceptibility of the DNA to cationic stimulated degradation.

#### 3.4. The effect of Mg<sup>2+</sup> on ISEL in rat liver nuclei

As shown above, end labelling provided a measure of the DNA strand breakage and the influence of Mg<sup>2+</sup> on this was investigated further. Increasing concentrations of Mg<sup>2+</sup> caused an increase in the level of ISEL with a maximum peak mean fluorescence at 8 mM Mg<sup>2+</sup>, followed by a decrease at 16 mM Mg<sup>2+</sup> (Fig. 4B). These results were in good agreement with the electrophoresis data described in Fig. 3, because as the DNA was cleaved into smaller fragments there would be a proportionate increase in the number of strand breaks and hence a higher level of ISEL. This conclusion was further supported by the data obtained after nuclei were incubated with a fixed concentration of Ca<sup>2+</sup> (200 μM) and varying Mg<sup>2+</sup> concentrations (Fig. 4A). A marked increase (10-fold) in the intensity of the green fluorescence was observed as compared to that produced by Mg<sup>2+</sup> alone (see

Fig. 4A,B). This increased ISEL corresponded to the increase in both the 30–50 kbp and oligonucleosomal fragments. The data demonstrated that Ca<sup>2+</sup> and Mg<sup>2+</sup> together produced a far greater amount of DNA strand breakage than did Mg<sup>2+</sup> alone.

#### 3.5. Time dependence of Mg<sup>2+</sup>-dependent DNA cleavage

The electrophoresis studies suggested that the Mg<sup>2+</sup>-dependent degradation of DNA progressed through a series of intermediate steps which generated successively

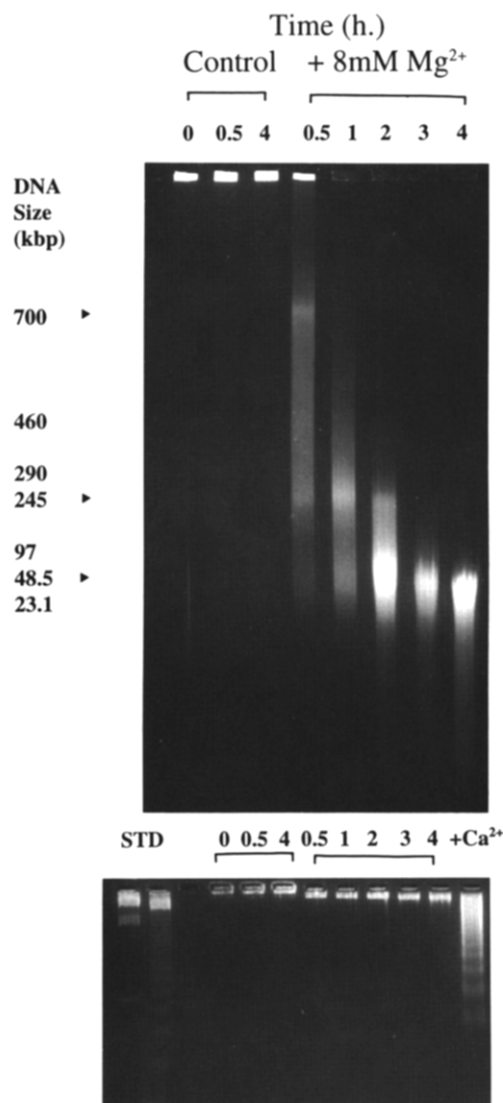


Fig. 5. Time course for Mg<sup>2+</sup> induced DNA fragmentation in rat liver nuclei as determined by conventional agarose gel and field inversion gel electrophoresis (FAGE). Rat liver nuclei were prepared and incubated at 37°C for 0–240 min with and without 8 mM Mg<sup>2+</sup> and at the indicated times the reactions were terminated and the DNA fragmentation patterns determined by FAGE (upper panel) and agarose gel electrophoresis (lower panel). Control lanes: nuclei incubated without magnesium for 0, 0.5 and 4 h, respectively; the remaining lanes show the effect of 8 mM Mg<sup>2+</sup> after 0.5, 1, 2, 3 and 4 h, respectively. A nuclei preparation showing DNA laddering with 8 mM Mg<sup>2+</sup> and 400 μM Ca<sup>2+</sup> is shown in the last lane of the agarose gel. The kbp marker positions are shown as ▶.

smaller sized fragments. This possibility was investigated further by following the time course of  $Mg^{2+}$ -dependent degradation. After 30 min the DNA was partially degraded into  $\geq 700$  kbp and 200–250 kbp fragments (Fig. 5), and by 1 h, the 200–250 kbp fragments predominated. Within 2 h, most of the latter were degraded into 30–50 kbp sized fragments and at 3 and 4 h only the 30–50 kbp fragments were observed. Significantly, there was no evidence of internucleosomal cleavage (lower panel, Fig. 5).

#### 4. Discussion

Endonuclease cleavage of DNA into nucleosomal fragments, which are detected as a DNA 'ladder' on agarose gel electrophoresis, has long been regarded as the biochemical 'hallmark' of apoptosis [2]. However, recent studies in this [10] and other laboratories [11] have shown that in thymocytes, there is an initial DNA cleavage into  $\geq 700$ , 200–300 and 30–50 kbp fragments, prior to internucleosomal cleavage. The size of the large fragments are similar to the 'loops' (30–50 kbp) and rosettes (200–300 kbp), which have been suggested by Filipinski et al. [17] to be essential components of higher order chromatin structure. A fuller understanding of these mechanisms/enzymes catalysing the formation of large fragments is of fundamental importance to the study of apoptosis. In this respect, studies on isolated nuclei provide an ideal model to investigate this phenomenon.

The results in this study show how a combined approach using conventional agarose gel electrophoresis, FIGE and ISEL can be used to investigate the DNA fragmentation process. Initially, the DNA was degraded into  $\geq 700$  kbp sized fragments which were subsequently cleaved in a  $Mg^{2+}$  concentration-, time-dependent and stepwise manner into 200–250 kbp and 30–50 kbp fragments (Figs. 1,5). When this process was catalysed by  $Mg^{2+}$  alone, no internucleosomal cleavage was observed (Figs. 1,2,5). Also,  $Ca^{2+}$  on its own was ineffective in stimulating the formation of large fragments or internucleosomal degradation (Figs. 1,3). However, low levels of  $Ca^{2+}$  (10  $\mu M$ ) in the presence of  $Mg^{2+}$  (4 mM) accelerated or potentiated the formation of large fragments and ultimately at higher  $Ca^{2+}$  concentrations produced internucleosomal cleavage (Fig. 1). This produced a much greater number of strand breaks as assessed by ISEL (Fig. 4A). The data obtained with the end labelling technique (Figs. 3,4.) demonstrated the potential of this technique to provide both a semi-quantitative assay of DNA cleavage but also to discriminate between nuclei with different ploidy. Interestingly, both diploid and tetraploid nuclei had the same susceptibility to cation stimulated endonuclease activity.

The stepwise nature of the degradation process was highlighted by the time course experiments (Fig. 5). The progression with 8 mM  $Mg^{2+}$  involved an initial cleavage

to the large  $\geq 700$  kbp fragments. Subsequently, these were cleaved into 200–250 kbp fragments and then ultimately to the 30–50 kbp fragments. In the absence of calcium there was no further cleavage. The data supported the idea that the breakdown of the DNA was an ordered process which was related to DNA structure.

The addition of  $Ca^{2+}$  (Figs. 1–4) in the presence of magnesium appeared to have two effects. Firstly, it enhanced the production of the 30–50 kbp fragments from the larger  $\geq 700$  and 200–250 kbp fragments, and secondly it activated internucleosomal cleavage. Both of these effects may be produced by the same enzyme/mechanism which is activated by magnesium but is accelerated or facilitated in the presence of  $Ca^{2+}$ . Alternatively, DNA fragmentation could be as a result of two separate and distinct processes or enzymes. In this context, it is of interest that Wyllie et al. [21], using a plasmid cutting assay have shown that a constitutive nuclease extracted from the nuclei of thymocytes, which are particularly susceptible to apoptosis can be activated by  $Mg^{2+}$  and that this activity is further facilitated by the addition of  $Ca^{2+}$ . Regardless of the mechanism involved, it is clear that we must reappraise the role of  $Ca^{2+}$  in the apoptotic process. Previously, the absence of DNA laddering in apoptotic cells [10–14] was interpreted as evidence that internucleosomal cleavage was an 'end stage' or 'downstream event'. Clearly, from our data this only occurs at moderately high calcium concentrations or at a late stage (time) in the process. However, it is equally clear that low levels of the  $Ca^{2+}$  in conjunction with  $Mg^{2+}$  will catalyse the rapid and the irreversible degradation of the DNA into large fragments and this may be an early event in the initiation of the apoptotic process.

It should be emphasised that our results provide information on the constitutive nucleases of isolated rat liver nuclei and strictly speaking this is not apoptosis. However, as discussed earlier the fragmentation patterns produced in isolated nuclei are similar to those observed in thymocytes induced to undergo apoptosis by topoisomerase II inhibitors [11,17] or by dexamethasone [10]. Thus, isolated nuclei would appear to be a valid experimental model for looking at the DNA fragmentation in apoptosis.

In conclusion, in isolated rat liver nuclei, the  $Mg^{2+}$  catalysed degradation of chromatin into large DNA fragments occurred without internucleosomal cleavage. The presence of  $Ca^{2+}$  stimulated this process and internucleosomal cleavage. Both processes may be carried out by the same enzyme(s) or alternatively DNA degradation in apoptosis may involve  $Mg^{2+}$ -dependent enzymes which are distinct from the  $Ca^{2+}/Mg^{2+}$ -dependent endonuclease(s). These results together with other studies with rat thymocytes [10,11] and isolated rat thymocyte nuclei [20] demonstrate that this progressive, multi-step, degradation mechanism is a common feature of apoptosis.

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