Nuclear Events of Apoptosis In Vitro in Cell-free Mitotic Extracts: A Model System for Analysis of the Active Phase of Apoptosis

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Abstract. We have developed a cell-free system that induces the morphological transformations characteristic of apoptosis in isolated nuclei. The system uses extracts prepared from mitotic chicken hepatoma cells following a sequential S phase/M phase synchronization. When nuclei are added to these extracts, the chromatin becomes highly condensed into spherical domains that ultimately extrude through the nuclear envelope, forming apoptotic bodies. The process is highly synchronous, and the structural changes are completed within 60 min. Coincident with these morphological changes, the nuclear DNA is cleaved into a nucleosomal ladder. Both processes are inhibited by Zn^{2+}, an inhibitor of apoptosis in intact cells. Nuclear lamina disassembly accompanies these structural changes in added nuclei, and we show that lamina disassembly is a characteristic feature of apoptosis in intact cells of mouse, human and chicken. This system may provide a powerful means of dissecting the biochemical mechanisms underlying the final stages of apoptosis.

STRICT control of cell loss as well as cell proliferation is essential for the coordinated functions of different cell populations in complex multicellular organisms. Programmed cell death has been proposed to be a major regulatory feature of embryological development (Glucksmann, 1951; Saunders, 1966; Kerr et al., 1974; Yuan and Horvitz, 1990), establishment of immune self-tolerance (Shi et al., 1989; Smith et al., 1989; MacDonald and Lees, 1990), immune effector cell killing (Ucker, 1987), and regulation of cell viability by hormones and growth factors (Cowan et al., 1984; Martin et al., 1988; Williams et al., 1990; Kyprianou and Issacs, 1988). Circumvention of the cell death program may contribute to neoplastic transformation (Tsujimoto et al., 1985; Hockenbery et al., 1990; Alnemri et al., 1992) and viral pathogenesis (Henderson et al., 1991; Gregory et al., 1991; Levine et al., 1993). Clearly, a better understanding of the process of programmed cell death may provide insights into mechanisms involved in the control of cell life span.

Programmed cell death may be considered as a two-phase process. In the first phase, cells commit themselves to enter the death pathway in response to a variety of stimuli. Cells in this stage may be considered to be in a "latent" phase of programmed cell death, since they may appear overtly normal and healthy even though they are committed to death. This latent phase is extremely variable, ranging from a few hours to days. Ultimately, however, the cells enter the "active" phase of programmed cell death, in which they undergo a dramatic series of morphological and physiological changes that culminate in death within an hour or so. Early studies of the active phase of cell death identified a characteristic morphologic evolution of dying cells, termed apoptosis. Apoptosis begins with condensation of nuclear chromatin at the nuclear periphery, follows with blebbing of the nuclear and cytoplasmic membranes, and culminates in the fragmentation of residual nuclear structures into discrete membrane-bounded apoptotic bodies (Kerr, 1971; Wyllie et al., 1980; Allen, 1987; Kerr et al., 1987).

Apoptosis occurs in many different cell types and in response to many different stimuli, of which a partial list includes DNA damage, withdrawal (or addition in some cases) of hormones of growth factors, inappropriate expression of genes that stimulate cell cycle progression, and viral infection. Several of these factors have in common the property that they alter the cell cycle, and it has been proposed that apoptosis primarily reflects a cell cycle perturbation (Ucker, 1991; Sen and D'Incalci, 1992). In some experimental systems, apoptotic death occurs only after cells complete the cycle and pass through mitosis (Kung et al., 1990; Kruman et al., 1991). This, and the observation that cell killing by cytotoxic T lymphocytes is accompanied by solubilization of the nuclear lamins (Ucker et al., 1992), have led to the proposal that apoptosis corresponds to an aberrant mitosis (Ucker, 1991).

It has proven to be relatively difficult to study the biochemical basis for the dramatic changes in cyto-architecture that

occur during the active phase of apoptosis. Not only do cells enter the active phase with little synchrony, but this phase of apoptosis is relatively brief compared to the length of the latent period. As a result, apoptotic cells tend to be encountered among larger numbers of viable cells. Thus, the underlying biochemical activities driving the morphological changes and leading to cell death remain largely undetermined. For example, the most extensively studied biochemical event in apoptosis is the cleavage of nuclear DNA, often visualized as a characteristic nucleosomal ladder (Wyllie, 1980; Duke et al., 1983; Arends et al., 1990; Kyprianou et al., 1988). However, the role, if any, of the nuclease in cell killing remains controversial as examples of apoptosis in the apparent absence of nuclease activity have been described (Cohen et al., 1992; Ucker et al., 1992).

In a previous study of mitotic chromosome condensation, we prepared highly concentrated cell-free extracts from mitotic DU249 chicken hepatoma cells (Langlois et al., 1974) that promoted chromatin condensation in cell nuclei isolated from a variety of cell types and species (Wood and Earnshaw, 1990). We report here the surprising result that the changes undergone by nuclei in those extracts correspond not to mitotic chromosome condensation, but instead to the nuclear changes that occur during the active phase of apoptosis. When nuclei are added to the extracts, the chromatin collapses against the nuclear periphery and becomes hypercondensed into spherical domains that are extruded through the nuclear envelope. At the same time, the nuclear lamina is dissassembled and the DNA is fragmented into a nucleosomal ladder. These changes, including disassembly of the nuclear lamina, accurately mimic the characteristic features of apoptosis in intact cells. Thus, these extracts may provide a powerful system with which to dissect the biochemical mechanisms underlying nuclear events during the final active phase of apoptosis.

Materials and Methods

Cell Culture

Chicken DU249 hepatoma cells (Langlois et al., 1974) were grown in RPMI 1640 plus 10% FBS in monolayer or suspension culture. HeLa S3 cells were grown in suspension culture in RPMI 1640 plus 5% calf bovine serum.

Preparation of S/M Extracts

S/M extracts were prepared essentially as described previously (Wood and Earnshaw, 1990). Briefly, chicken DU249 cells (Langlois et al., 1974) were presynchronized in S phase with aphidicolin for 12 h, released from the block, and synchronized in mitosis with nocodazole for 3 h. Mitotic cells were obtained by selective detachment. (Fig. 1 *B* shows an electron micrograph section of one of the cells harvested for production of S/M extracts. This cell appears to be in a normal C-mitosis.) The cells were then washed with extract preparation buffer (50 mm Pipes, pH 7.0, 50 mm KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM cytochalasin B, and the following protease inhibitors: PMSF, chymopain, leupeptin, antipain, and pepstatin), and centrifuged in a small glass Dounce homogenizer (Kontes Glass Co., Vineland, NJ). After complete aspiration of the supernatant, the dounce was subjected to several cycles of freezing and thawing in a isopropanol:dry ice bath. During each thawing cycle, the cells were further disrupted by grinding with the pestle. The cell lysate was then centrifuged for 3 h at 150,000 *g*, yielding a clear preparation of mitotic cytosol. This extract was diluted to a protein concentration of 8 mg/ml (2–3-fold) with mitotic dilution buffer (MDB)[1] (Newport and Spann, 1987): 10 mm Hepes, pH 7.0, 40 mm β-glycerophosphate, 50 mm NaCl, 2 mM $MgCl_2$ 5 mM EGTA, 1 mM DTT, and supplemented with an ATP-regeneration system containing final concentrations of 2 mM ATP, 10 mM creatine phosphate, and 50 μg/ml creatine kinase. The reaction mixture contained 10 μl of diluted extract and up to 10^6 nuclei. Reactions were incubated at 37°C and at the time indicated 1.5 μl were stained with the DNA-specific dye DAPI and the membrane probe DHCC (3,3′ dihexyloxacarbocyanin; Eastman Kodak Co., Rochester, NY).

Interphase extracts were made from DU249 cells logarithmically growing in monolayer or suspension culture by the same protocol as S/M extracts. Cells from monolayer cultures were harvested by trypsinization.

When cells were stained with DAPI and scored before making extracts, ≥95% of the cells appeared to be in mitosis. When adherent cells were used for extract preparation, dead cells were discarded by one round of selective detachment performed 2 h before the final harvesting of the culture. In interphase extracts (which had no activity in the in vitro assay) up to 2% of the cells appeared to be in apoptosis in some experiments. Thus, we believe it highly unlikely that a minor population of contaminating cells could explain the apoptotic activity of our extracts.

To examine the effects of various divalent cations on the in vitro reaction, extracts were dialyzed at 4°C for 3 h against 1,000 vol of MDB lacking EGTA using Spectra/Pore 1 dialysis membranes with molecular weight cutoff 6,000–8,000 D.

Purification of the Interphase Nuclei

HeLa nuclei were prepared essentially as described previously (Wood and Earnshaw, 1990). The cells were harvested by centrifugation, washed twice with PBS, once with NB (10 mM Pipes, pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, plus proteinase inhibitors), and then resuspended in 10 vol of NB. The cells were allowed to swell on ice for 20 min and then were gently lysed with a Dounce homogenizer. The resulting homogenate was layered over 30% sucrose in NB. The nuclei were pelleted by centrifugation at 800 *g* for 10 min, washed once in NB, were resuspended at a concentration 5×10^7 nuclei/ml. Nuclei were either used immediately after preparation or were stored up to one week at −20°C in the following buffer: 10 mM Pipes, pH 7.4, 80 mM KCl, 20 mM NaCl, 250 mM sucrose, 5 mM EGTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, proteinase inhibitors, plus 50% glycerol (Newport and Spann, 1987). Nuclei were washed twice in MDB before addition to S/M extracts. Fig. 1 *A* shows an electron micrograph of one of these nuclei in thin section. The chromatin appears to be highly dispersed, and the nuclear architecture looks completely different from that of nuclei undergoing morphological apoptosis (Fig. 3).

Microscopy

Cells or nuclei to be processed for EM were centrifuged at 400 *g*, fixed with 2% glutaraldehyde in D-PBS (6.46 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 0.137 M NaCl, 2.68 mM KCl, 0.492 mM $MgCl_2$, 0.68 mM $CaCl_2$) for 1 h, washed in 0.1 M cacodylate, pH 7.4, and fixed with 0.1% OsO_4 in 0.1 M cacodylate for 15 min. After fixation, the cells were washed with distilled water and stained with 3% aqueous uranyl acetate for 2 h. The cells were then dehydrated in graded ethanol and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Thin sections were placed on copper grids and stained with uranyl acetate and lead citrate.

Fluorescence microscopy was performed using an Olympus Vanox microscope equipped with a filter set from Chromo Technology Corporation (Burlington, VT). Images were acquired using a DAGE SIT camera controlled by the Hyperscope image analysis program (Perceptics Corp., Nashville, TN). The montages shown in the figures were assembled using Adobe Photoshop and printed using a GCC Colorfast printer.

Isolation of DNA from Nuclei following Incubation in S/M Extracts

S/M and interphase extracts were diluted to a final protein concentration of 6–8 mg/ml with MDB buffer and supplemented with the ATP-regeneration system 1.5–3 × 10^6 HeLa nuclei were mixed with 30 μl of extract or extraction buffer and incubated for various times at 37°C (1 h for the experiment shown in Fig. 5 and 7, and 0, 15, 30, 45 and 70 min for the experiment shown in Fig. 4). At the end of this incubation, one tenth of each aliquot was stained with DAPI and examined by fluorescence microscopy. The remainder of the reaction mixture was then centrifuged at 10,000 rpm in a microfuge for 5 min and total nuclear DNA was extracted from pellets as described (Ausubel et al., 1991).

1. *Abbreviations used in this paper*: APA, apoptosis promoting activity; MDB, mitotic dilution buffer.

Indirect Immunofluorescence of Nuclear Lamins

Cells were fixed with 4% formaldehyde and lamins were subsequently localized by indirect immunofluorescence (Wood and Earnshaw, 1990). A mAb to chicken lamin B2 (Lehner et al., 1986) (gift of E. Nigg, ISREC, Epalinges s/Lausanne, Switzerland) was used for DU249 cells. An affinity-purified polyclonal antibody to human lamin A (gift of Brian Burke (Harvard Medical School, Boston, MA)) was used to stain SN12C cells and thymocytes.

Cell Viability after Release from the Block Induced by Aphidicolin or Nocodazole

The DU249 cells were synchronized as described in Fig. 8 (protocols 1-3). Mitotic cells were harvested by selective detachment, washed in culture medium by centrifugation, and resuspended in suspension culture at a concentration of 10^5 cells/ml. At the indicated time, an aliquot of the suspension was removed and the cells pelleted at 300 *g* for 5 min and resuspended in PBS. To measure viability, 3 vol of the suspension were mixed with 1 vol of 0.4% solution of trypan blue (Sigma Immunochemicals, St. Louis, MO) and Trypan blue-stained cells were counted using light microscope. To measure percentage of apoptotic and mitotic cells the cells in PBS were fixed with 4% formaldehyde, stained with DAPI, and then examined in the fluorescence microscope. Mitotic cells were recognized based on the distinctive morphology of the condensed chromosomes. Cells with DNA staining appeared as multiple round shaped and evenly stained bodies were scored as apoptotic. At least 400 cells were counted for each measurement.

Depletion of cdc2/cdk2 from S/M Extracts Using p9 Beads

To deplete the extracts of cdc2/cdk2 we used p9 beads, which consist of chicken p9 protein covalently bound to cyanogen bromide (CnBr)-activated Sepharose. The beads were kindly provided by R. Golsteyn and E. Nigg (ISREC). p9 protein is a vertebrate homologue of $p13^{suc1}$ protein. BSA coupled to the Sepharose was used for control depletions. We developed a protocol of depletion that minimizes dilution of the extracts. The beads were incubated for 1 h with 35% BSA at 4°C, washed five times with MDB, and then resuspended in equal amount of MDB. 100 ml of this suspension was placed into a micro-filtration unit (No. SJHVL04NS; Millipore Corp., Milford, MA). After the removal of buffer by centrifugation at 14,000 rpm in a microfuge for 5–10 s (the beads remain on top of the filter) 50 μl of the extract was added. The suspensions were continuously rotated in the micro-filtration unit at 4°C for 1 h. Subsequently, the mitotic extract was collected by centrifugation as described above. (Extracts passed through the filter while beads remained trapped.) Extracts obtained in this way were used for H1 kinase and morphological assays. A portion of this extract was subsequently used for a second round of depletion using a fresh aliquot of the beads. All assays were performed with equivalent concentrations of extract protein (15 mg/ml for the first round, 13.5 mg/ml for the second round).

H1 Kinase Assay

p9 or BSA beads were washed five times with MDB supplemented with 500 mM NaCl and 0.1% Tween 20 and were resuspended in 200 μl of MDB. 10 μl of this suspension or 1 μl of the extracts at the protein concentrations given above were mixed with 80 μl H1 assay buffer (MDB supplemented with 0.5 mg/ml histone H1, type III-S [Sigma Immunochemicals], 3 μM PKI [cAMP-dependent protein kinase inhibitor; Sigma Immunochemicals], 80 μM ATP, and 160 μCi/ml ^{32}Pg-ATP). After incubation for 10 min at room temperature the reaction was stopped by addition of an equal volume of SDS sample buffer and the samples were boiled for 3 min. 20 μl of each sample were loaded on 15% SDS-polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue, dried and exposed to x-ray film. An average time of exposure was 1 h.

MPM-2 Staining

Murine thymocytes treated with 0.1 μM dexamethasone were pre-attached to Adhesio-Slides (MM Developments, Ottawa, Canada). SN12C cells were grown on coverslips. The cells were fixed with 4% formaldehyde and processed for indirect immunofluorescence using MPM-2 antibody diluted 1:200 and biotinylated horse anti-mouse secondary antibody with streptavidin-Texas red. Images were acquired as described above with gain settings equal for all taken pictures. Normal mouse serum (Sigma Immunochemicals) was used instead of MPM-2 as a control for staining of thymocytes. No first antibody was used as a control when SN12C cells were stained.

Results

A Cell-free Extract from Chicken Hepatoma (DU249) Cells Causes Isolated Nuclei to Undergo Morphological Changes Characteristic of Apoptosis

We made cell-free extracts from mitotic chicken hepatoma (DU249) cells that were subjected to a sequential S phase/M phase synchronization protocol. Cultures were first arrested in S phase for 10–12 h with aphidicolin (1 μg/ml), released

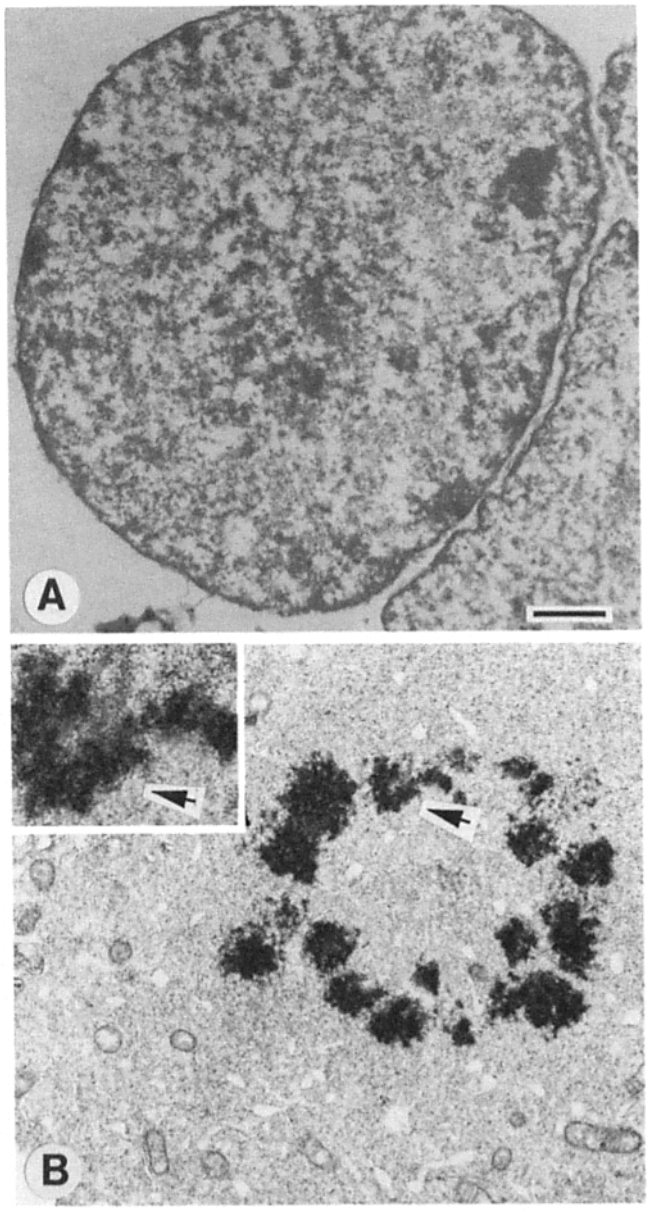


Figure 1. Ultrastructure of cells containing latent apoptosis-promoting activity and of nuclei undergoing apoptosis in vitro. (*A*) Thin section of an isolated nucleus before addition to the S/M extracts. The chromatin is relatively dispersed in nuclei isolated by this protocol. (*B*) Thin section of DU249 cell treated and harvested as for extract production. This cell appears to be in normal mitosis, with chromosomes containing readily observed trilaminar kinetochores (*arrows*). The inset shows a higher magnification view of the chromosome indicated by the arrow. Bar, 1 μm.

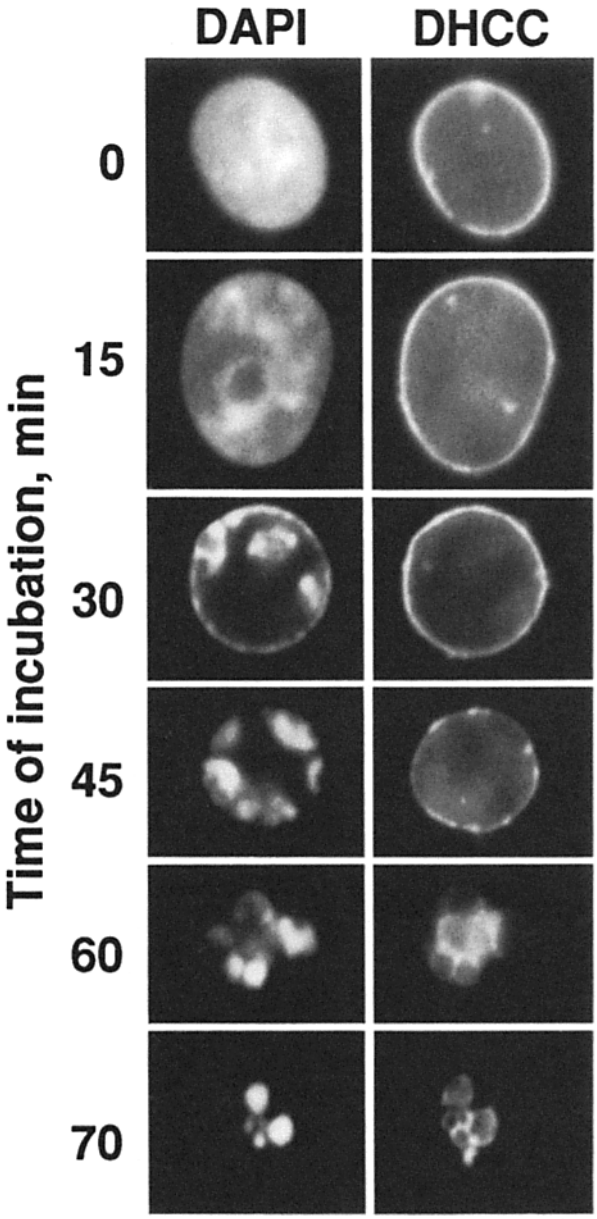


Figure 2. Time course of the morphological changes observed upon addition of nuclei to S/M extracts. Aliquots of HeLa nuclei added to S/M extracts were removed at the indicated times and examined in the fluorescence microscope. The left panels show DAPI stain for DNA. The right panels show the DHCC stain for membrane.

from the block and allowed to progress through the cycle for 6 h, and then treated with nocodazole and allowed to accumulate in mitosis for a further 3 h. Highly concentrated cytoplasmic extracts were then prepared from these mitotic cells (Wood and Earnshaw, 1990). We refer to extracts prepared from cells synchronized in this way as "S/M" extracts.

Purified nuclei added to S/M extracts underwent a dramatic morphological change within one hour at 37°C. After ~15 min, they began to shrink as the chromatin condensed around the nuclear periphery (Fig. 2). With increasing time of incubation, the peripheral chromatin ring began to condense into discrete masses. Finally, the chromatin masses appeared to bleb off from the nuclear surface, emerging as pebble like bodies that stained uniformly with the fluorescent DNA dye DAPI (Fig. 2, *60* and *70* min). Staining with the fluorescent lipophilic dye DHCC indicated that the nuclear membrane persists throughout this process: even the condensed DNA "pebbles" are surrounded by a lipid membrane.

The morphological process was extremely efficient: all nuclei added to the extracts underwent these structural transformations, and they did so in a highly synchronous manner. Up to 10^5 nuclei could be induced to undergo this morphologic process per microliter of extract (equivalent to cytoplasm from ~10^6 mitotic cells). The process was temperature dependent, with the chromatin condensation requiring 2 h at room temperature, and not being observed at all at 4°C, even after a 3-h incubation.

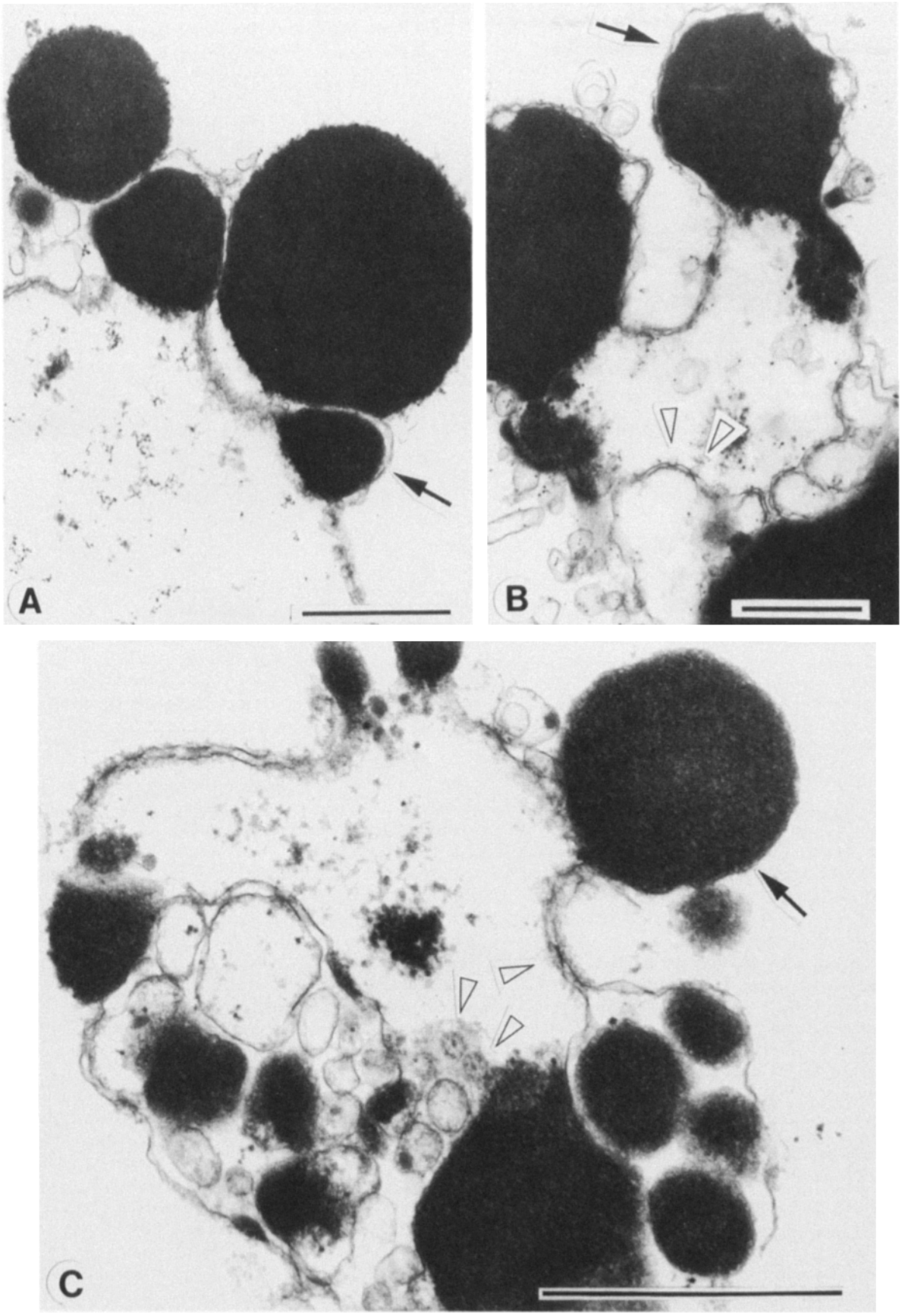
Ultrastructural Analysis of Nuclei Undergoing Morphological Apoptosis In Vitro

When the condensed nuclei were fixed with glutaraldehyde and examined by thin section EM, they showed a striking similarity to nuclei of cells undergoing apoptosis. The chromatin was segregated into hyper-condensed domains that assumed a sharply defined spherical shape during the later stages of the process. These spherical domains often appeared to be in the process of budding outwards through the nuclear envelope (Fig. 3). Chromatin domains in the process of budding were surrounded by a membrane that appeared to be continuous with the remnant of the nuclear envelope visible between condensed chromatin domains (Fig. 3, *solid arrows*) as predicted from their staining properties with DHCC (Fig. 2). That the domains were actually budding outwards through the nuclear envelope is supported by the observation that when nuclei were left in the extracts for longer periods of time, the DNA was released into the extract as individual highly condensed "pebbles" (see Fig. 5).

The portion of the residual nuclear envelope not involved in blebbing typically retained its original morphology. Structures resembling nuclear pore complexes could be observed to bridge the membrane of this residual envelope between blebs containing condensed chromatin (Fig. 3, *open arrowheads*). The nuclei often retained residual nucleolar structures in which the granular and dense fibrillar components were readily visible (not seen in Fig. 3). These residual nucleoli stained intensely with propidium iodide (not shown).

The morphological transformations induced in isolated nuclei by S/M extracts in vitro strongly resemble the characteristic changes of apoptosis undergone by intact cells undergoing programmed cell death in vivo. This is illustrated in Fig. 4, in which we compare the morphology of apoptosis induced in vivo in human GM3798 lymphoblastoid cells fol-

Figure 3. Ultrastructure of HeLa nuclei undergoing apoptosis in vitro. Note the hypercondensation of the chromatin into membrane-enclosed apoptotic bodies that are seen in the process of extruding through the nuclear envelope. The solid arrows indicate regions where the membrane surrounding the apoptotic bodies is clearly visualized. The open arrowheads indicate nuclear pores, three of which are seen en face in *C*. This concentration of nuclear pores between apoptotic bodies looks strikingly similar to a published image of a human



breast lobular cell undergoing apoptosis (Wyllie et al., 1980). We note, that as in the case described by Wyllie et al. (1980), the nuclear pores remain concentrated between apoptotic bodies, and appear to be excluded from the membrane overlying the condensed chromatin. Bar, 1 μm.

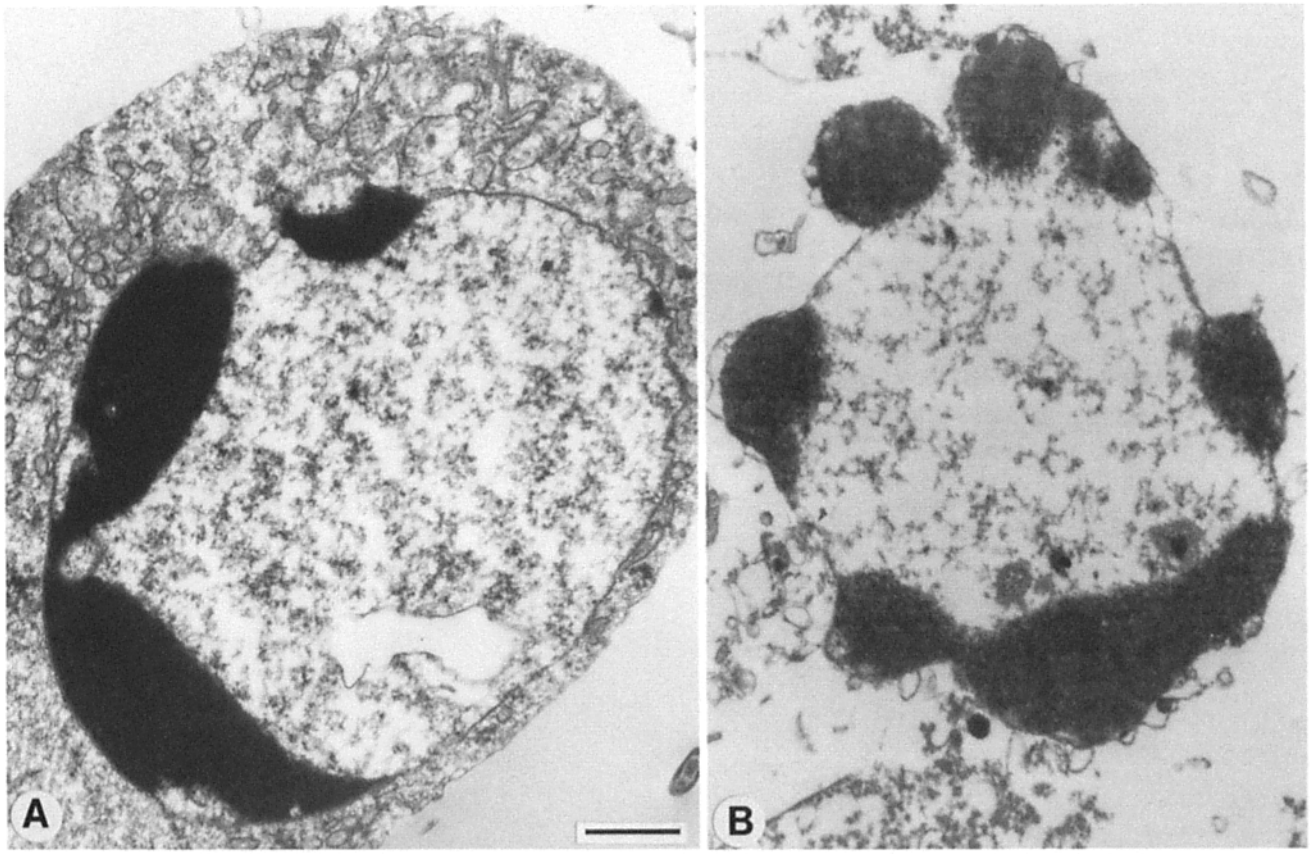


Figure 4. Similar morphology of nuclei from human GM3798 lymphoblastoid cells undergoing apoptosis in vitro and in vivo. (*A*) Apoptosis in living GM3798 cells was induced by addition of 0.1 μM dexamethasone to growing cultures. This cell entered apoptosis 6 d after addition of the drug. (*B*) Nuclei isolated from GM3798 cells were placed in S/M extract and incubated for 1 h at 37°C before fixation for EM.

lowing addition of dexamethasone (Fig. 4 *A*), with the morphology of nuclei isolated from these cells following incubation in S/M extracts (Fig. 4 *B*). In both cases, the DNA is highly condensed against the margins of the nuclei, which retain only an amorphous fibrillar network throughout their interior. The only slight difference seen in these experiments is in the detailed shape of the condensed DNA domains, which typically appear slightly more spherical in nuclei incubated in S/M extracts. This difference is relatively minor, and might be due to the presence of a supporting cytoplasmic matrix surrounding the nucleus in the intact cells.

Given this strong resemblance between the morphological events of apoptosis in vivo and in S/M extracts, we will refer to the events of chromosome condensation and blebbing that occur in S/M extracts as "morphological apoptosis."

Morphological Apoptosis In Vitro Is Accompanied by Internucleosomal Cleavage of the DNA

To assess the validity of our in vitro system as a model for apoptosis in vivo, we have examined whether this in vitro process shares other common features with the events of apoptosis in cells. One widely observed property of apoptotic cells is the cleavage of the DNA into fragments at sites separated by the internucleosomal spacing (Wyllie, 1980; Duke et al., 1983; Arends et al., 1990). Although exceptional cases have been described where apoptotic death occurs in the apparent absence of nuclease (Cohen et al., 1992; Ucker et al., 1992), cleavage of the nuclear DNA is typically considered to be a hallmark of apoptosis.

The morphological changes in added nuclei appeared to occur simultaneously with the cleavage of the chromatin into a nucleosomal ladder. Agarose gel electrophoresis of total DNA prepared from HeLa nuclei after incubation in S/M extracts revealed a "ladder" of fragments with a spacing of about 175 bp (Fig. 5, lanes *S/M*). In contrast, nuclei incubated in extract buffer alone or in extracts prepared from asynchronous cell cultures failed to display any significant DNA cleavage (Fig. 5, lanes *IE*). Fragmentation of the DNA was detected as soon as the DNA was observed to move to the nuclear periphery (Fig. 6).

Neither the morphological changes nor DNA fragmentation in added nuclei showed an absolute requirement for the presence of free Ca^{2+} in our extracts (the extraction buffer contains 5 mM EGTA and addition of a further 5 mM EGTA to the reaction mixture had no effect). This was initially surprising, since a Ca^{2+} or Ca^{2+}/Mg^{2+}-activated endonuclease has been proposed to be responsible for the cleavage of the nuclear DNA during apoptosis (Wyllie, 1980; Arends et al., 1990). However, others have suggested that the apoptotic nuclease is more closely related to DNase I or DNase II, neither of which requires Ca^{2+}.

Morphological apoptosis was strictly dependent on the presence of Mg^{2+} in S/M extracts. Both the morphological

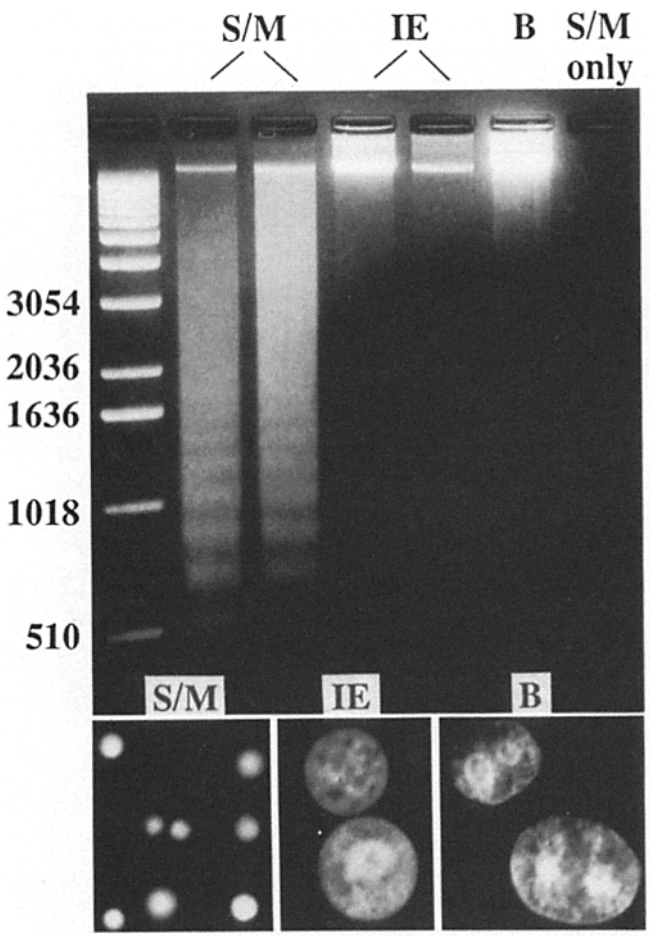


Figure 5. Exposure of nuclei to S/M extracts results in cleavage of the DNA into a nucleosomal ladder not seen when nuclei are incubated in control extracts. Nuclei were added a final concentration of 40,000/μl to either of two independent S/M extracts (lanes *S/M*), two independent extracts made from cultures in exponential growth (lanes *IE*), or buffer (lane *B*). After a 60-min incubation at 37°C, nuclei were centrifuged. Total nuclear DNA was then isolated from the pellet and subjected to electrophoresis in a 1% agarose gel. Lane S/M only shows a gel of the S/M extract without added nuclei, confirming that this extract does not contain detectable DNA. The three panels shown at the bottom indicate the morphology of the nuclei prior to isolation of the DNA. All three panels are shown at identical magnifications: the small structures seen in panel S/M (nuclei treated with S/M extract) are dispersed apoptotic bodies seen when the in vitro reaction goes to completion.

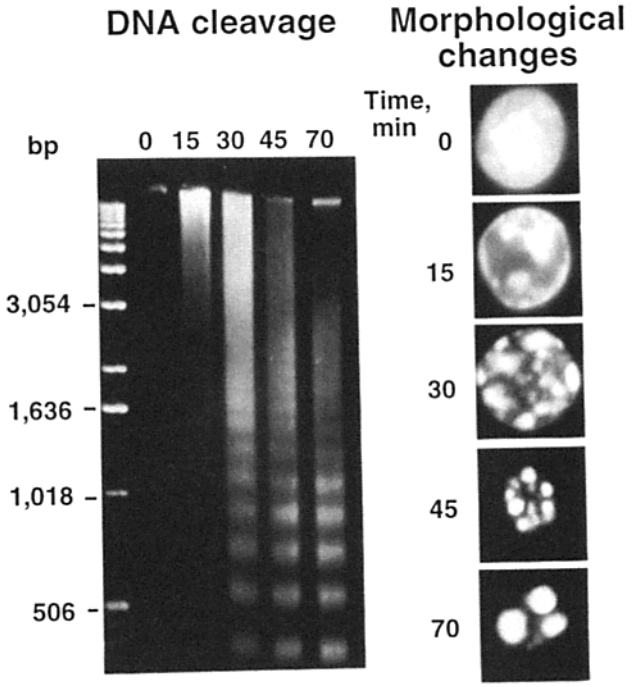


Figure 6. Morphological changes in nuclei are accompanied by internucleosomal DNA cleavage in S/M extracts. HeLa nuclei were incubated with S/M extract for the indicated period of time. An aliquot of each sample was then stained with DAPI to show morphological changes. Total nuclear DNA was extracted from the rest of the sample and visualized by agarose gel electrophoresis.

reaction and nuclease activity were abolished by the addition of 5 mM EDTA (Fig. 7). Furthermore, dialysis of extracts against a buffer lacking Mg^{2+} caused their irreversible inactivation.

To distinguish between the involvement of various known endonucleases in the DNA degradation we examined the sensitivity of the process to divalent cations other than Mg^{2+}. For these experiments, extracts were dialyzed against extract preparation buffer minus EGTA (but containing 2 mM Mg^{2+}). Such dialyzed extracts were as active as non dialyzed extracts in promoting both the morphological apoptosis and nuclease cleavage reactions, provided that Mg^{2+} was present during dialysis (Fig. 7). As a control for the effect of increasing the overall level of divalent cations, dialyzed extracts were supplemented with additional $MgCl_2$ up to a total of 7 mM with no detectable effect on their activity. In contrast, addition of 2 mM $MnCl_2$ to dialyzed extracts abolished the nuclease activity (Fig. 7). The chromatin appeared to condense, but was not concentrated into sharply defined domains, causing the nuclei to adopt a disordered appearance distinct from any of the intermediates observed during the normal condensation reaction (compare Figs. 6 and 7).

Zn^{2+}, which has previously been shown to inhibit apoptosis and the associated nuclease activity in vivo (Duke et al., 1983; Cohen and Duke, 1984; Flieger et al., 1989; Cohen et al., 1992) was highly inhibitory both to the morphological transformation and nuclease activities in S/M extracts. Addition of $ZnCl_2$ at concentrations as low as 0.5 mM appeared to arrest morphological apoptosis at an early stage in the pathway (compare Figs. 6 and 7), but had almost no effect on the activity of the nuclease (Fig. 7). $ZnCl_2$ at 2 mM abolished both the morphological transformation and the nuclease activity. At 1 mM $ZnCl_2$, the morphological transformation was abolished, but significant levels of nuclease activity remained. Inhibition of the nuclease activity by $ZnCl_2$ suggests that this activity may be distinct from DNase I, which can use $ZnCl_2$ as a cofactor (albeit poorly) (Junowicz and Spencer, 1973).

The ability of low levels of added $ZnCl_2$ to block morphological apoptosis while leaving the activity of the nuclease basically intact suggests that the extract may contain a Zn^{2+}-sensitive component in addition to the nuclease. This component (which could be a second nuclease different

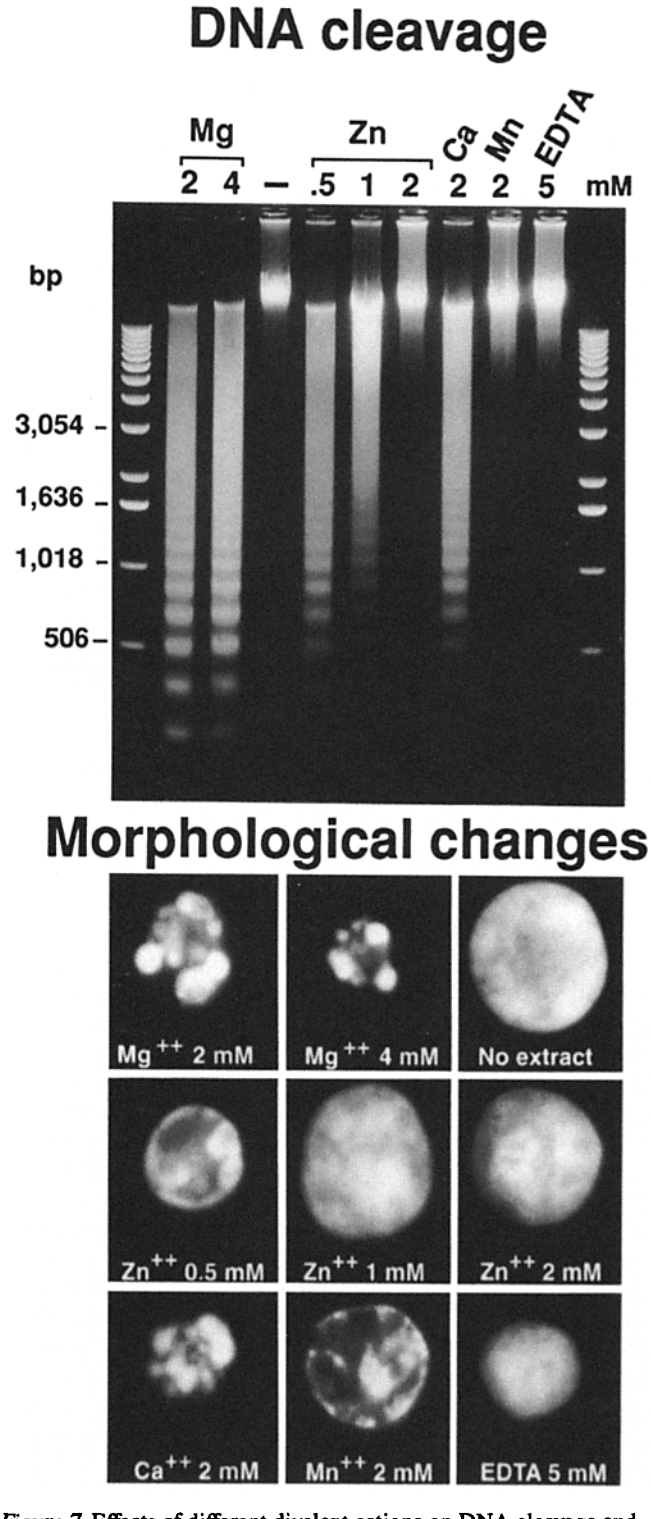


Figure 7. Effects of different divalent cations on DNA cleavage and morphological apoptosis in S/M extracts. The condensation reaction was performed using dialyzed extracts from which EGTA had been removed. Different divalent cations were then added to the reaction mixtures. Every sample had 2 mM $MgCl_2$ plus the indicated concentration of divalent cation. After 1 h of incubation at 37°C an aliquot of each sample was stained with DAPI to register morphological changes. Total nuclear DNA was then extracted from the rest of the sample and visualized by agarose gel electrophoresis.

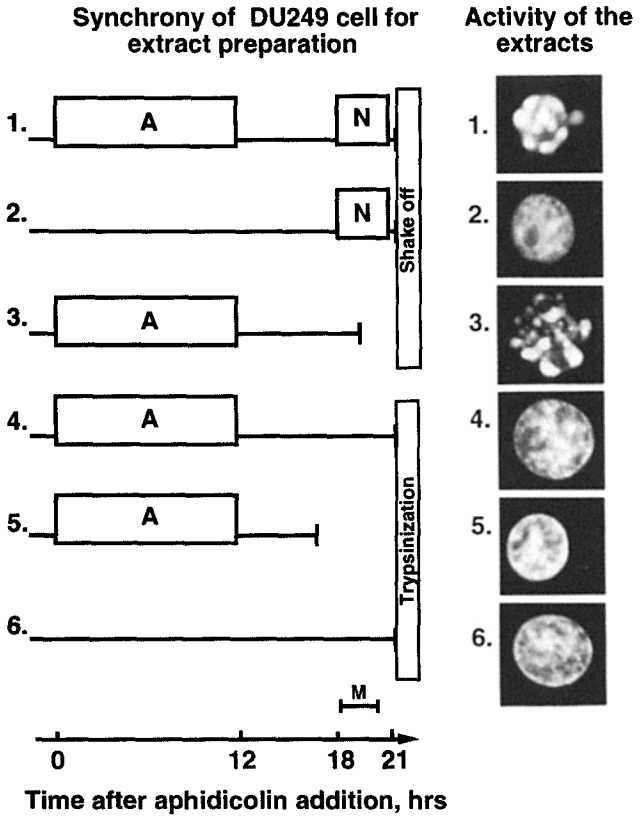


Figure 8. The ability of cell-free extracts to promote apoptosis in vitro requires both a prior aphidicolin treatment and harvesting of the cells while they are in mitosis. Chicken hepatoma DU249 cells were processed by several different protocols and used for the preparation of concentrated extracts. In each case the protocol is indicated at the left, and the morphology of nuclei added to the corresponding extract is shown at the right. Protocol 1 is the standard protocol used in preparation of S/M extracts. In protocol 2, cells from a log-phase culture were incubated with nocodazole for 3 h to accumulate mitotic cells, which were then obtained by selective detachment. This is a true mitotic extract. (It should be noted that we would not expect to observe true mitotic chromatin condensation at the high concentration of nuclei used for this experiment (40,000/μl) (Newport and Spann, 1987).) In protocol 3, cells were treated with aphidicolin and then allowed to continue their progress through the cell cycle for 7 h, at which point many cells were in mitosis. These mitotic cells were harvested by selective detachment and used for extract production. In protocol, 4 cells were treated with aphidicolin and then allowed to continue their progress through the cell cycle for 9 h, by which point most of the culture had passed through mitosis. These cells were harvested by trypsinization and used for extract production. In protocol 5, cells were treated with aphidicolin and then allowed to continue their progress through the cell cycle for 4.5 h, at which point the culture had not yet entered mitosis. These cells were harvested by trypsinization and used for extract production. In protocol 6 cells growing in log-phase culture were harvested by trypsinization and used for extract preparation.

in sensitivity to Zn^{2+}) is apparently essential for the chromatin condensation and blebbing in added nuclei.

We do not know if the nuclease responsible for cleavage of the nuclear DNA is supplied in soluble form by the extract, or is an endogenous nuclear enzyme that is somehow

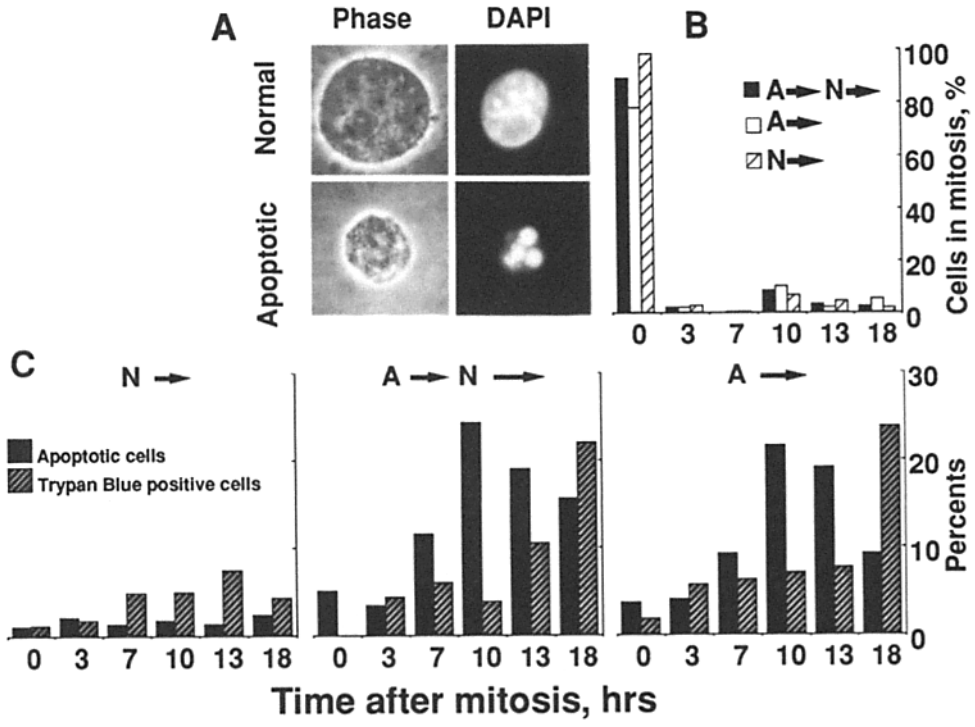


Figure 9. The cells synchronized for the extract preparation die after passing mitosis if released from the cell cycle block. DU249 cells were synchronized by different protocols, harvested by shakeoff in mitosis, transferred to drug-free medium, and allowed to resume progression through the cell cycle. (*A→N→*) The protocol used for S/M extract preparation (protocol 1, Fig. 8); N→ the cells were blocked with nocodazole for 2 h without prior aphidicolin treatment (protocol 2, Fig. 8); A→ the cells were blocked with aphidicolin as for the extract preparation, released and harvested by shakeoff in mitosis without treatment with nocodazole (protocol 3, Fig. 8). At the indicated time, the number of mitotic, apoptotic, interphase, and dead (trypan blue positive) cells was scored. (*A*) A photograph of a cell scored as apoptotic together with an example of a normal cell. (*B*) The change in the number of mitotic cells after transferal to drug-free medium. (*C*) The percentage of apoptotic and trypan blue positive cells after transferal of the cultures to drug-free medium.

activated by the extract, or both. S/M extracts contain soluble endonuclease activity, however this shows a different sensitivity to added divalent cations than does the nuclease responsible for cleavage of the nuclear DNA. The extract nuclease activity is partly inhibited by 2 mM $ZnCl_2$, but is unaffected by similar levels of $MnCl_2$ when assayed by cleavage of plasmid DNA (data not shown). This suggests either that the nuclear DNA is cleaved by an endogenous nuclease that is sensitive to both Zn^{2+} and Mn^{2+} or that Mn^{2+} specifically blocks the action of the extract nuclease on nuclear substrates.

A Link between Expression of Apoptosis Promoting Activity and Entry of Aphidicolin-treated DU249 Cells into Mitosis

The data presented thus far suggest that S/M extracts contain an activity (or activities) that cause added nuclei to undergo the morphological events of apoptosis in vitro. For convenience, we will refer to this activity as apoptosis promoting activity (APA)[1]. We stress, however, that the composition of APA is at present undetermined, and that APA could consist of several activities (including a nuclease) acting in concert.

We have considered two possible explanations for the presence of APA in the S/M extracts. First, these extracts are made from mitotic cells, and APA might be an intrinsic feature of mitotic DU249 cells. Alternatively, the presence of APA in the S/M extracts might be a consequence of some aspect of our sequential S/M phase synchronization protocol. of examine this issue, we assessed the apoptosis-promoting activities of extracts from chicken hepatoma DU249 cells obtained in several different ways.

To examine the possible connection between the mitotic state of the cells and the presence of APA activity, we prepared extracts from cells arrested in mitosis without any aphidicolin presynchrony (Fig. 8, protocol *2*). Cells in exponential growth were exposed to nocodazole for 3 h, and highly enriched populations of mitotic cells (≥95%) were obtained by selective detachment. Extracts prepared from these mitotic cells lacked APA, and did not induce either degradation of the nuclear DNA (data not shown) or the hypercondensation and blebbing of chromatin. Thus, entry into mitosis alone was not sufficient to induce extractable APA in DU249 cells. This observation eliminates the possibility that APA is an artifactual consequence of our method of extract preparation that is apparent only when the cells used to prepare extract are in mitosis.

We next examined the possibility that the aphidicolin treatment might be responsible for the presence of APA in S/M extracts. We exposed DU249 cells to aphidicolin for 10–12 h (as in the preparation of S/M extracts), removed the drug to allow cells to progress through the cycle, and prepared extracts at varying time intervals. Extracts were made from cells treated in this way and then harvested 1.5 h before mitosis, during mitosis, or 1 h after mitosis. Extracts made from cells harvested either before or after mitosis lacked APA (Fig. 8, protocols *5* and *4*, respectively). In contrast, extracts made from mitotic cells harvested by selective detachment had APA and induced morphological apoptosis in added

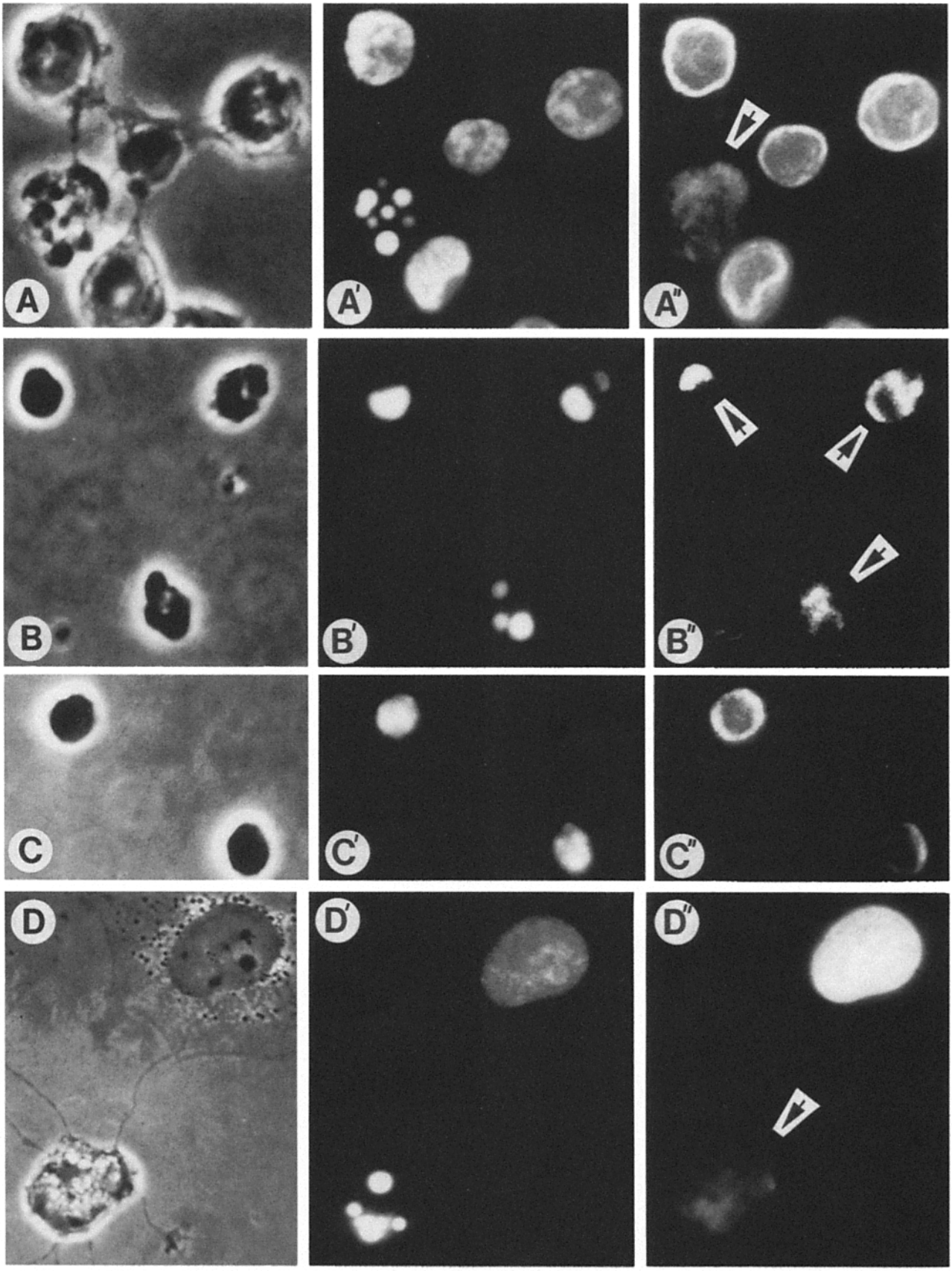


Figure 10. The nuclear lamina is dissembled in cells undergoing apoptosis in culture. (A′–A″) Chicken hepatoma DU249 cells, which occasionally undergo spontaneous apoptosis in culture. (B′–B″) Thymocytes from C57Bl/6 mice following exposure to 1 μM dexamethasone in culture. (C′–C″) A portion of these cells that were cultured in the absence of dexamethasone. (D–D″) Human SN12C renal carcinoma cells undergoing apoptosis in culture in response to the sequential exposure to camptothecin and tumor necrosis factor. (A–D) phase contrast; (A′–D′) DAPI staining of the DNA; (A″–D″) visualization of lamin A using an affinity-purified antibody directed against a peptide determinant. The arrowheads in the latter panels indicate cells undergoing morphological apoptosis.

nuclei (Fig. 8, protocol *3*). Thus, all that was required for APA induction was pretreatment of DU249 cells with aphidicolin followed by entry into mitosis.

The DU249 from Which S/M Extracts Are Prepared Are Committed to Apoptotic Death, but Appear Normal at the Time of Harvesting

We were initially surprised to note that "mitotic" extracts prepared from DU249 cells synchronized by the S/M protocol caused added nuclei to undergo the morphological events of apoptosis, since these cells appeared to be arrested in a normal C metaphase (a prometaphase-like state induced by the disassembly of microtubules (Molè-Bajer, 1958) at the time of extract preparation, as shown in Fig. 1 *B*. Normal condensed mitotic chromosomes with morphologically intact kinetochores were observed. The organization of the cytoplasm appeared normal, albeit slightly vacuolated. Furthermore, when these cells were removed from nocodazole and replated, they completed mitosis and reattached to the substratum (data not shown).

We were concerned that cells that were apparently not undergoing apoptosis themselves could give rise to extracts that induce the morphological events of apoptosis in added nuclei. Since the latent phase of apoptosis can extend for many hours or even days, we examined the fate of these cells over a longer period of time. Cells synchronized exactly as described for the production of extracts (Fig. 8, protocol *1*) were released from the mitotic block and allowed to proceed into the subsequent cell cycle. At intervals of 3–4-h aliquots were removed from the cultures and scored for three characteristics: (*a*) presence of a mitotic phenotype; (*b*) presence of an apoptotic phenotype (defined by the condensation of the chromatin into discrete "pebbles" that stain evenly with DAPI) (Fig. 2) (this was confirmed by EM; data not shown); and (*c*) viability, as defined by the ability to exclude trypan blue dye.

The synchronized DU249 cells appeared to undergo apoptosis 7–10 h after exiting from the mitosis in which they would normally have been harvested for production of S/M extracts (Fig. 9 *A* and *C*). This corresponded roughly to the time at which a portion of the population passed through a second mitosis (Fig. 9 *B*). As is normally the case for apoptotic cell death, the cells did not become permeable to trypan blue until some time after the chromatin adopted a typical condensed apoptotic state (Sheridan et al., 1981). In some experiments, up to 35% of the cells showed the DNA condensed into apoptotic bodies, and up to 45% of the cells eventually became Trypan blue positive.

Two control experiments were performed. In the first, cells subjected to mitotic arrest with nocodazole without prior aphidicolin treatment (Fig. 8, protocol *2*) were obtained by selective detachment following a 2 hour treatment with the drug. When transferred to fresh drug-free medium, these cells readily reestablished themselves in culture, divided and continued to grow (Fig. 9 *C*). In a second experiment, cells were subjected to aphidicolin arrest, transferred into fresh medium without drug to enable them to proceed through the cycle, and then harvested in mitosis by selective detachment (Fig. 8, protocol *3*). These cells, which were not exposed to nocodazole, subsequently underwent apoptosis on schedule with their counterparts that were subjected to the aphidicolin/nocodazole treatment (Fig. 9 *C*).

Thus, synchronization protocols that resulted in the presence of APA in mitotic extracts made from DU249 cells induced cell death 7–10 h after mitosis if cells were allowed to continue into the next cell cycle.

Nuclear Lamina Disassembly Is a Characteristic Feature of Apoptosis In Vivo

We have used indirect immunofluorescence and immunoblotting with mono-specific antibodies to confirm that nuclear lamins A and B_2 are undetectable in nuclei that have been incubated in S/M extracts (Wood and Earnshaw, 1990; and data not shown). Thus if these extracts induce morphological apoptosis in added nuclei, as we believe, then lamina disassembly should be a characteristic feature of apoptotic cell death in vivo. We have confirmed this prediction, and have demonstrated that the nuclear lamina is disassembled

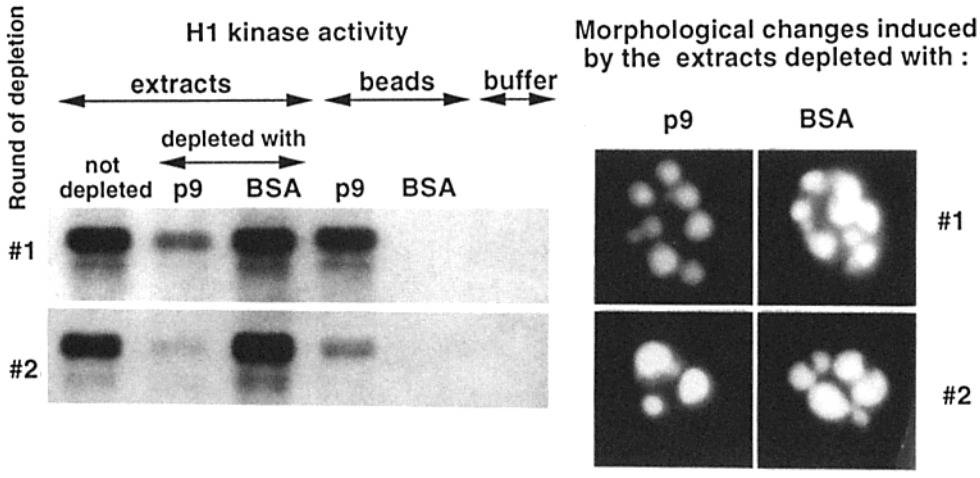


Figure 11. Depletion of H1 kinase activity from S/M extracts has no effect on the ability of the extracts to induce morphological apoptosis in added nuclei (*Left*) S/M extracts were incubated with p9/agarose or BSA/agarose as described in Materials and Methods, and the supernatant and beds subsequently assayed for H1 kinase activity. Two successive rounds of depletion were carried out on the same extract. The lanes are as follows: (*extracts*: *not depleted*, starting level of H1 kinase assay in the original S/M extract; *p9*, H1 kinase activity remaining in extract following centrifugation; *BSA*, H1 kinase activity remaining in extract following centrifugation); (*beads*: *p9*, H1 kinase activity bound to the p9/agarose beads; *BSA*, H1 kinase activity bound to the BSA/agarose beads); and (*buffer*: H1 kinase activity in the extract buffer alone). (*Right*) Representative morphology of nuclei that had been incubated for 1 h at 37°C in extracts depleted with either 1 (*#1*) or 2 (*#2*) rounds of absorption with p9 or BSA agarose.

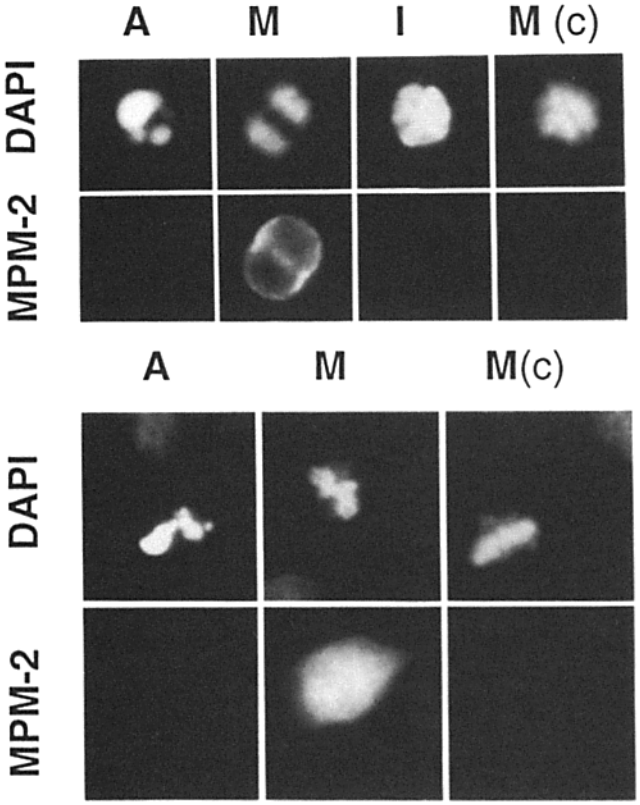


Figure 12. Human and murine cells in apoptosis are not stained with the mitosis-specific antibody MPM-2. (*Top*) Murine thymocytes were induced to undergo apoptosis following exposure to 0.1 μM dexamethasone. The top panels show DAPI-stained images of the DNA of representatives cells in apoptosis (*A*), mitosis (*M*), or interphase (*I*). The bottom panels show staining of these same cells with the MPM-2 antibody. Panel M(c) shows a mitotic cell stained with an irrelevant murine mAb. (Mitotic cells were found at low frequency in this crude cellular preparation from macerated thymus.) (*Bottom*) Human renal carcinoma (SN12C) cells were induced to enter apoptosis by sequential exposure to camptothecin and tumor necrosis factor. Panels are labeled as in the upper portion of the figure.

in three cell types undergoing apoptotic death in culture. The first two of these, murine thymocytes treated with dexamethasone (Wyllie, 1980) (Fig. 10, *B* and *C*) and apoptotic human renal cancer cells (SN12C) treated with camptothecin and tumor necrosis factor (Nelson, W. G., manuscript in preparation) (Fig. 10 *D*) undergo programmed death with all of the classical features of apoptosis. When populations containing apoptotic cells were stained with a monospecific antibody recognizing lamin A (gift of B. Burke), this protein was either found to be dispersed throughout the cytoplasm, or was undetectable altogether in apoptotic cells.

We also observed cells that appeared to be spontaneously undergoing apoptosis in cultures of DU249 chicken hepatoma cells that had been permitted to grow past log phase. When these cells were stained with a monoclonal antibody monospecific for chicken lamin B_2 (gift of E. Nigg, ISREC) (Fig. 10 *A*), this protein was found to be either dispersed throughout the cytoplasm, or undetectable.

This demonstration that both A and B type lamins disassemble during apoptosis suggests strongly that the nuclei of cells undergoing apoptosis lack a nuclear lamina. These results are in agreement with a recent study revealing that the nuclear lamins of virally transformed NIH 3T3 cells are solubilized as the cells undergo apoptotic death following exposure to cytotoxic T lymphocytes (Ucker et al., 1992), a process that shares many common properties with apoptosis.

Morphological Apoptosis in S/M Extracts Does Not Require H1 Kinase Activity

The demonstration that APA is present only when extracts are prepared from pre-apoptotic DU249 cells while they are in mitosis, and that the nuclear lamin is solubilized during apoptosis together raise the possibility that $p34^{cdc2}$ kinase might be an essential component of APA. If true, this would give substantial support to the notion that apoptosis is a form of aberrant mitosis.

However, two experiments argue strongly that $p34^{cdc2}$ kinase is not an essential component of APA. First, we attempted to adsorb $p34^{cdc2}$ kinase from the extracts using p9:agarose beads (gift of Roy Goldsteyn and Erich Nigg, ISREC). p9 is the vertebrate homologue of $p13^{suc1}$, and these beads have the capacity to bind both $p34^{cdc2}$ and $p33^{cdk2}$. Complete removal of $p34^{cdc2}$ kinase with these beads is technically very difficult, however in two successive rounds of adsorption we were able to lower the level of H1 kinase in S/M extracts by up to 95% (Fig. 11). These depleted extracts were as active in carrying out morphological apoptosis as were control extracts that had been incubated with BSA/agarose.

The second experiment involved staining cells undergoing apoptosis in vivo with mAb MPM-2, which is specific for a number of mitotic phosphoproteins (gift of Potu Rao, University of Texas, M. D. Anderson Cancer Center, Houston, TX). We have examined human SN12C (renal carcinoma) and murine thymocytes undergoing apoptosis in response to camptothecin/TNF or dexamethasone, respectively. In neither case was staining above background observed in any cell undergoing apoptosis (Fig. 11). In contrast, mitotic cells observed in both preparations were stained intensely.

Together, these observations argue strongly that apoptotic cells are not in an aberrant mitosis-like state. This is particularly intriguing, given the observation that the nuclear lamina is disassembled in these very cells as they undergo apoptosis (see Fig. 10).

Discussion

A Cell-free System Accurately Reproduces the Active Phase of Apoptosis In Vitro

The cell-free extract system described here causes isolated nuclei to undergo the morphological transformations characteristic of apoptosis with a much higher degree of synchrony than is available in current systems for studying apoptosis in vivo. In living cells, the decisive factor that identifies a morphologic process as apoptotic is its ultimate termination in cell death. Of course this decisive event cannot occur in a cell-free system such as ours. However a number of dramatic correspondences between the changes undergone by isolated nuclei in S/M extracts and by nuclei in cells during apoptosis in vivo lead us to conclude that the in vitro process described

here is a useful model for the active phase of apoptosis. These correspondences are listed below.

(*a*) When nuclei are added to S/M extracts, the chromatin collapses outwards against the nuclear envelope and subsequently undergoes a dramatic hypercondensation into sharply defined spherical domains. EM reveals these domains to be more tightly and uniformly condensed than mitotic chromosomes. The final morphology, the sequence of these events and the time required for their completion are all highly similar to the pattern of events that occurs during apoptosis in living cells. (*b*) The reorganization and condensation of the chromatin is accompanied by cleavage of the DNA into fragments that are multiples of the spacing between nucleosomes. As discussed below, the activation of a nuclease is recognized as a hallmark of apoptosis in most characterized systems. (*c*) The morphological transformations and nuclease activity are both inhibited by low concentrations of Zn^{2+}. Zn^{2+} has been shown to inhibit apoptosis in murine thymocytes and to inhibit cellular nucleases thought to be involved in apoptosis (Duke et al., 1983; Cohen and Duke, 1984; Flieger et al., 1989; Cohen et al., 1992). (*d*) The terminal phase of the process undergone by nuclei in S/M extracts is an active fragmentation by blebbing apart into membrane-enclosed vesicles. This resembles the final structural changes seen during apoptosis in cultured cells and is completely unlike any effect on nuclear structure described to date in cell-free systems derived from mitotic or meiotic cells (Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985; Suprynowicz and Gerace, 1986; Newport and Spann, 1987; Högner et al., 1988; Nakagawa et al., 1989). (*e*) The changes in structural organization undergone by nuclei in S/M extracts are accompanied by the disassembly of the nuclear lamina. This observation from the cell free system has been shown to apply to intact cells as well, and we have demonstrated here that cells from mouse, human and chicken all disassemble their nuclear lamina during apoptosis in vivo. This disassembly of the nuclear lamina is unusual, in that it is not accompanied by disassembly of the nuclear membrane or nuclear pore complexes. A similar situation is seen in intact tissues, where concentrations of nuclear pore complexes between condensed chromatin domains have been seen in cells undergoing apoptosis in vivo (Wyllie et al., 1980). (*f*) Preliminary experiments indicate that if S/M extracts are introduced into live cells in culture by scrape loading (McNeil et al., 1984), the cells undergo a process resembling apoptosis within 90 min (Cole, S., Y. Lazebnik, and W. C. Earnshaw, unpublished observations).

These similarities between the morphological and biochemical processes occurring in our cell-free extracts and during the active phase of apoptosis in live cells provide compelling evidence that this system mimics the active phase of programmed cell death by apoptosis.

The apoptotic activity present in S/M extracts can be explained by the fact that the cells from which the extracts are made are in the latent phase of apoptosis even though they appear based on morphological observation to be in a typical C mitosis. As has been described for some other cell types in culture (Kung et al., 1990), DU249 cells do not tolerate the cell cycle perturbation induced by lengthy treatment with aphidicolin. If returned to culture, a substantial percentage of these cells undergo apoptotic death 7–10 h after they exit mitosis. When examined by EM, the S/M cells have numerous small lacunae in their cytoplasm. This "moth-eaten" appearance has been suggested to be a hallmark of cells in the initial stages of apoptosis (Allan et al., 1992).

For convenience of discussion we will refer to the dramatic changes in nuclear structure that occur in S/M extracts as "morphological apoptosis", and to the sum of the activities present in S/M extracts that bring about morphological apoptosis in added nuclei as APA (Apoptosis Promoting Activity). APA may be comprised of several components, one (or more) of which may be a nuclease.

The Nuclear Lamina Is Disassembled during Apoptosis In Vitro and In Vivo

The morphological changes that occur when nuclei are added to S/M extracts are accompanied by disassembly of the nuclear lamina (Wood and Earnshaw, 1990). Our realization that the extracts induce morphological apoptosis led us to examine whether lamina disassembly also occurs in living cells as they undergo the active phase of apoptosis. As shown in Fig. 10, lamina disassembly occurred in three different cell types from three different species (murine thymocytes, human endothelial carcinoma, chicken hepatoma) as these cells underwent apoptosis in culture.

Solubilization of the nuclear lamina in mitosis is a consequence of the phosphorylation of the lamin subunits by one or more protein kinases (Gerace and Blobel, 1980; Gerace and Burke, 1988; Peter et al., 1990; Nurse, 1990). At present we do not know if a similar mechanism of lamina disassembly is involved during apoptosis (particularly in murine thymocytes, which undergo programmed cell death in the G_0 phase of the cell cycle). In contrast to mitotic cells, which maintain a depolymerized lamin pool for lamina reassembly at the end of mitosis (Gerace and Blobel, 1980), apoptotic cells appear to ultimately degrade their lamin subunits (Fig. 10; and data not shown). Degradation of the lamins was also noted during cell death induced by a variety of anti-cancer drugs (Kaufmann, 1989). This lamin cleavage during apoptosis may help to serve to make nuclear disassembly irreversible.

Why disassemble the nuclear lamina during apoptosis? The answer to this could lie in the involvement of the nuclear lamina in the organization of the chromatin in interphase nuclei. It has long been thought that chromatin may interact with the nuclear periphery (Gerace and Burke, 1988), and published evidence suggests that the lamina may bind DNA (Lebkowski and Laemmli, 1982; Ludérus et al., 1992). Interactions between the chromosomes and the nuclear envelope might need to be severed before the dramatic reorganization and blebbing of the DNA into hypercondensed apoptotic bodies can occur. One way to accomplish this would be to disassemble the lamina.

A Ca^{2+}-independent, Zn^{2+}-sensitive Nuclease Fragments the Nuclear DNA during Morphological Apoptosis in S/M Extracts

Although digestion of the nuclear DNA into a nucleosomal ladder is one of the hallmarks of apoptotic cell death, relatively little is known about the nuclease (or nucleases) involved in this process. Early studies of apoptosis in thymocytes implicated a Ca^{2+} or Ca^{2+}/Mg^{2+}-activated nuclease in the chromatin degradation (Wyllie, 1980; Cohen and Duke,

1984; McConkey et al., 1989; Arends et al., 1990). DNA cleavage was inhibited by Zn^{2+} (Cohen et al., 1992), as was the apoptotic process itself in these cells (Duke et al., 1983; Cohen and Duke, 1984; Flieger et al., 1989). Later studies proposed that the relevant nuclease is either DNase I (Ucker et al., 1992; Peitsch et al., 1993) or DNase II (Barry and Eastman, 1993). It is important to note, however, that although these nuclease activities were detected in cells that can undergo apoptosis, no direct evidence was presented to demonstrate that these particular nucleases are actually the ones activated during apoptosis.

Morphological apoptosis in isolated nuclei added to S/M extracts occurs simultaneously with the digestion of the nuclear DNA into a nucleosomal ladder. The nuclease responsible for this digestion requires divalent cations but not Ca^{2+}. As in the studies with intact lymphocytes (Cohen et al., 1992) both the morphological apoptosis and nuclease activity are inhibited by Zn^{2+} and Mn^{2+}. This pattern of sensitivity to added divalent cations resembles that shown by endonuclease R from HeLa cells (Gottlieb and Muzczka, 1990). However endonuclease R is unlikely to be responsible for the cleavage of nuclear DNA in S/M extracts, since it exhibits a significant degree of sequence specificity (for runs of G residues), at least on naked DNA (Gottlieb and Muzczka, 1990).

Our data are consistent with the involvement of a novel nuclease in the cleavage of nuclear DNA in these extracts. Alternatively, other factors may interact with one of the known enzymes to modulate its sensitivity to divalent cations (see Peitsch et al., 1993).

Is Apoptosis an Aberrant Form of Mitosis?

Several similarities between apoptosis and mitosis led to the suggestion that these two processes are somehow related (Ucker, 1991). These included the dramatic condensation of the chromatin during apoptosis (Kerr, 1971), the timing required for the active phase of apoptosis, the observation that the final cellular fragmentation in apoptosis, like cytokinesis, is an actin-dependent process (Cotter et al., 1992), and the observation that the nuclear lamina is disassembled during cell killing by cytotoxic T lymphocytes (Ucker et al., 1992). This notion is supported in the experiments reported here, where we find that pre-apoptotic DU249 cells give rise to extracts containing APA only if they are harvested in mitosis.

Despite this list of similarities, a number of observations suggest that the link between mitosis and apoptosis, if any, is complex. For example, many cell types that undergo apoptosis presumably do so directly from the G_0 phase. In addition, we have observed three salient morphological differences between mitosis and apoptosis. (*a*) The nucleolus does not disassemble during the initial stages of apoptosis, either in vivo (Allen, 1987; Ucker et al., 1992), or in isolated nuclei added to cell-free extracts with APA activity (data not shown). Nucleolar disassembly is an early event in mitosis. (*b*) Although the nuclear lamina is disassembled both in vivo and in vitro during apoptosis, the nuclear membrane persists. Apoptotic bodies remain surrounded by membrane both in vivo and in our in vitro system. (*c*) When the hypercondensed apoptotic chromatin domains in apoptosis are compared to mitotic chromosomes by EM, the two are seen to be quite different. The apoptotic chromatin achieves a homogeneous packing that appears significantly more dense than that in mitotic chromosomes.

Given this evidence both for and against the idea of a similarity between apoptosis and mitosis, how do we explain the link between mitosis and the presence of APA in S/M extracts? One possibility is that maturation promoting factor (MPF; a complex of active $p34^{cdc2}$ kinase with a cyclin cofactor; Nurse, 1990) might be a component of APA. Our experiments render this highly unlikely. First, we have shown that depletion of 90–97% of the H1 kinase activity from S/M extracts has no effect on their ability to cause added nuclei to undergo morphological apoptosis. Second, we have shown that apoptotic cells do not stain with the MPM-2 mAb, a widely used marker for mitotic cells (Davis et al., 1983). These results do not, however rule out the possibility that a transient activation of one of the mitotic kinases or phosphatases leads to the activation of APA.

Alternatively, the apparent link between APA and mitosis could reflect some technical aspect of the way in which S/M extracts are made. If the extractability of an essential component of APA were to vary as a function of the cell cycle, the activity might be present before, during and after mitosis, but only appear in extracts made from mitotic cells. For instance, an essential component of APA might be sequestered tightly in the nucleus and be removed along with the DNA during the centrifugation steps when extracts are made from interphase cells. Such a component might be liberated into the cytosol following nuclear breakdown at mitosis.

A similar mechanism could operate during apoptosis in vivo in DU249 cells. The cell cycle perturbation caused by exposure of DU249 cells to aphidicolin could result in the accumulation or activation of several components of APA. If these were somehow kept apart in the cell (perhaps through compartmentalization), then the APA would remain latent. During mitosis, the dramatic reorganization of cytoarchitecture might abolish this compartmentalization, thus permitting the various components to associate with one another and produce active APA. This model may provide an explanation for the observation that the aphidicolin-treated DU249 cells die by apoptosis at about the time they enter the next mitosis (although we cannot explain why they transit the first mitosis after aphidicolin treatment successfully). In other studies, several cell types have been shown to undergo apoptotic death only after they pass mitosis (Kung et al., 1990; Kruman et al., 1991; Warters, 1992).

Clearly the connection between apoptosis and mitosis is complex: present data do not support the idea that apoptosis is simply an aberrant form of mitosis. One key to understanding the link between these two processes will be determination of the composition of APA.

What Is APA?

Although we have chosen to use a single term, APA, to denote the apoptosis promoting activity present in our cell-free extracts, we believe it entirely likely that APA consists of a number of activities. These may include specific nucleases and proteases in addition to enzymes, such as kinases and phosphatases, that are more frequently associated with the regulation of cell cycle transitions. If, as we have argued, S/M extracts provide an accurate model system for the final active phase of apoptosis, then the characterization of APA is clearly of great interest, since this activity causes a unique

morphological transformation that culminates in nuclear disintegration.

Although we have yet to isolate and characterize the components of APA, our experiments permit us to conclude that this activity must contain components in addition to the nuclease(s) responsible for digestion of the nuclear DNA. For example, our experiments suggest that S/M extracts contain a Zn^{2+}-sensitive factor that is required for the morphological events of apoptosis, but not for cleavage of the DNA. Treatment of extracts with low levels of Zn^{2+} (0.5 mM) caused the morphological apoptosis to become arrested at an early stage, but had little discernible effect on the activity of the endonuclease (see Fig. 7). The nuclease and morphological apoptosis activities could also be separated based on their requirement for ATP. Depletion of ATP from the extracts with apyrase or addition of non-hydrolyzable analogs of either ATP or GTP completely abolished morphological apoptosis in added nuclei. Instead the chromatin adopted a disorganized threadlike appearance. Under these conditions the nuclear DNA showed a normal pattern of cleavage into a nucleosomal ladder, indicating that the nuclease does not require hydrolyzable ATP or GTP (data not shown). Our results are in agreement with recently published work showing that treatment of hepatocyte nuclei with nucleases in vitro cannot reproduce the characteristic pattern of chromatin condensation observed during apoptosis in vivo (Oberhammer et al., 1993).

In addition to the nuclease and the activities responsible for the hypercondensation of chromatin and blebbing of compacted chromatin domains through the nuclear envelope, APA also contains an activity that brings about the solubilization of the nuclear lamina. This activity was first identified in S/M extracts (Wood and Earnshaw, 1990), but we have shown here that lamin solubilization is a widespread phenomenon during apoptosis in vivo (see also Ucker et al., 1992). It is unlikely that nuclease treatment alone would result in solubilization of the nuclear lamina, since extensive nuclease digestion is used as an important step during the biochemical isolation of the pore complex/lamina fraction from isolated nuclei (Dwyer and Blobel, 1976).

It is our belief that the S/M extract system described here may provide a significant step forward towards the identification of the biochemical activities that drive the dramatic changes in nuclear structure during the active phase of apoptosis.

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