American Journal of Pathology, Vol. 136, No. 3, March 1990

Apoptosis

The Role of the Endonuclease

M. J. Arends, R. G. Morris, and A. H. Wyllie
From the Department of Pathology, University of Edinburgh Medical School, Edinburgh, United Kingdom

Cell death by apoptosis mediates several important physiologic and pathologic processes and appears to be intrinsically programmed. Its characteristic features are distinctive morphologic changes of nucleus and cytoplasm, along with cleavage of chromatin at regularly spaced sites. Here we study DNA organization and nuclear structure in apoptotic thymocytes to define the cleavage event and, by implication, the role of the responsible endonuclease. We show that in apoptosis, double-stranded cleavage of DNA generates two classes of chromatin fragments: 70% of DNA exists as long, H1-rich oligonucleosomes bound to the nucleus, and 30% comprises short oligonucleosomes and mononucleosomes, which are depleted in H1, enriched in HMG1 and 2, and not attached to the nucleus. This minority class probably derives from chromatin in a transcriptionally active configuration, which would allow better access to enzymes in the nucleoplasm, producing more complete digestion. The characteristic nucleolar morphology in apoptosis can also be explained in terms of cleavage of the transcriptionally active ribosomal genes, with conservation of the nucleolin-rich fibrillar center. The chromatin cleavage, nucleolar morphologic changes, and chromatin condensation were closely mimicked by micrococcal nuclease digestion of normal thymocyte nuclei in the presence of protease inhibitors. Thus, in apoptosis, selective activation of an endogenous endonuclease appears to be responsible not only for widespread chromatin cleavage but also for the major nuclear morphologic changes. (Am J Pathol 1990, 136:593–608)

Apoptosis is a process whereby cells die in a controlled manner, in response to specific stimuli, apparently following an intrinsic program.[1] It occurs in developmentally regulated cell death in the embryo,[2–4] in deletion of autoreactive T-cell clones during thymic maturation,[5,6] in senescence of neutrophil polymorphs,[7] and following removal of specific growth factors (eg, in lymphocytes deprived of IL-2[8] and prostatic and endometrial cells deprived of steroid hormones)[9–11] or the addition of physiologic regulatory hormones (eg, in lymphocytes exposed to glucocorticoids).[12,13] Apoptosis is also found in cells attacked by cytolytic T-lymphocytes (CTL) and natural killer (NK) cells[14–16] and in lymphoid cells exposed to moderate doses of ionizing radiation.[17,18] In all of these circumstances there is a series of strikingly similar morphologic changes, several of the most conspicuous occurring in the nucleus.[1,15] At the same time, nuclear DNA undergoes widespread cleavage at internucleosomal sites.[13,19]

The morphologic changes of apoptosis occur in three phases.[20] In the first, there is condensation of chromatin into crescentic caps at the nuclear periphery, nucleolar disintegration, and reduction in nuclear size. Shrinkage of total cell volume, increase in cell density, compaction of cytoplasmic organelles, and dilatation of endoplasmic reticulum are observed, but the mitochondria remain morphologically normal. In phase 2 (which may overlap with phase 1), there is budding and separation of both nucleus and cytoplasm into multiple, small, membrane-bound apoptotic bodies, which may be shed from epithelial surfaces or phagocytosed by neighboring cells or macrophages. In phase 3 there is progressive degeneration of residual nuclear and cytoplasmic structures. Possible intracellular signaling mechanisms in the initiation of apoptosis include influx of calcium ions[21,22] and altered expression of oncogenes c-*fos* and c-*myc*.[23–25] In some cell types the process appears dependent upon protein synthesis and can be abrogated by application of inhibitors shortly after the lethal stimulus.[26,27] Little is known, however, of the intracellular effector mechanisms in apoptosis and in particular of the regulation of DNA cleavage.

This work was supported by the Cancer Research Campaign.
MJA is in receipt of a Medical Research Council Training Fellowship.
Accepted for publication November 2, 1989.
Address reprint requests to M.J. Arends, Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK.

It is widely assumed that DNA cleavage is the result of endogenous endonuclease activity. A Ca^{2+} and Mg^{2+}-dependent endonuclease activity capable of cleaving chromatin at internucleosomal sites has been identified in nuclear preparations from normal (but apoptosis-prone) cells.[28] Identification of an enzyme specifically associated with programmed cell death would be of substantial interest, but to date there is little information on whether apoptosis involves appearance of new intranuclear nuclease activity (by synthesis or activation) or rearrangement of chromatin proteins to permit DNA cleavage by pre-existing enzyme. Nor is it clear to what extent the DNA cleavage is responsible for the striking morphologic changes in the apoptotic nucleus. The possibility that many other cytolytic enzymes may be involved coordinately has not been directly addressed.

This paper describes a more detailed analysis of the structure of the apoptotic nucleus than has been previously attempted. We have used rodent thymocytes treated with glucocorticoid as the experimental model, because large numbers of apoptotic cells can be prepared and purified *in vitro* in a form readily amenable to biochemical and structural analysis. The data from these approaches provide strong evidence that the structural changes in the nucleus in apoptosis are the direct result of a selective nuclease activation within the dying cells.

Materials and Methods

Preparation of Cells

Thymocytes were obtained from the thymus glands of previously untreated suckling Sprague-Dawley rats by teasing into suspension in medium. They were incubated at 2×10^7 ml at 37 C in Dulbecco's modification of Minimum Eagles Medium (MEM) supplemented with 10% heat-inactivated newborn calf serum. In some experiments, the cortical thymocytes were labeled *in vitro* by intrathymic injection under anesthesia, with approximately 50 μCi tritium-labeled thymidine (Amersham International, Amersham, UK), 48 hours prior to dissection. To induce apoptosis, the synthetic glucocorticoid methylprednisolone sodium succinate (Solumedrone, Upjohn, Crawley, UK) was added to cultures at 10^{-5} M. Lower concentrations of 10^{-6} M and 10^{-7} M were also effective.

Density separation of apoptotic from morphologically normal thymocytes was effected on step gradients of Percoll (Pharmacia, Uppsala, Sweden) made up in Hepes-buffered MEM as described.[12] Thymocytes were concentrated by centrifugation, loaded on 10-ml gradients, and centrifuged at 2000*g* for 10 minutes.

Lysis Procedures

1) *Isotonic lysis*, to separate nuclei from cytoplasm, was conducted in 100 mmol NaCl, 1.5 mmol $MgCl_2$, and 10 mmol TRIS pH 7.4 (SMT buffer) supplemented with 0.15% Nonidet P40 and 1 mmol phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 110*g* to pellet nuclei.
2) *Hypotonic lysis* (applied to whole cells or to nuclei prepared as above) was conducted in 5 mmol TRIS pH 7.4, 5 mmol ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, and 1 mmol PMSF. The lysate was centrifuged at 27,000*g* for 20 minutes to separate high- from low-molecular-weight chromatin.

Analytic Procedures

DNA was measured by the diphenylamine reaction,[29] after precipitation in 10% trichloroacetic acid (TCA), or was concentrated by precipitation in ethanol, extracted with chloroform, and studied by electrophoresis in 1.8% agarose gels including ethidium bromide.[30] In some experiments *oligonucleosomes* were fractionated on 12 ml 10% to 30% neutral sucrose gradients made up in 5 mmol TRIS pH 7.4, 1 mmol EDTA, and spun at 30,000 rpm for 16 hours in a Beckman SWTi rotor (Beckman, Warrington, UK) at 4 C before deproteination and electrophoresis.

Nucleoprotein in cell lysates was fixed in 1% neutralized formaldehyde.[31] In experiments using fixation, the isotonic lysis buffer above was modified by substituting 10 mmol triethanolamonium chloride buffer for TRIS to avoid inactivation of the fixative aldehyde groups. Fixed nucleoprotein was banded by isopyknic centrifugation in cesium chloride (initial refractive index 1.3785) at 40 C in a Beckman SW60 Ti rotor at 35,000 rpm for 68 hours. Nucleoprotein peaks were identified by tritium counts (in preparations made from animals injected intrathymically 2 to 3 days before death with labeled thymidine) or by fluorescence following addition of ethidium bromide to 1 μg/ml, using excitation and emission wavelengths of 540 nm and 610 nm, respectively. DNA fluorescence was distinguished from that of RNA by repeating the measurements after digestion for 60 minutes at 37 C with 0.75 mg/ml RNAse (Sigma, Poole, UK) rendered DNAse-free by heating to 100 C for 30 minutes.

Sucrose gradient fractionated oligonucleosomes were deproteinized and digested with S1-nuclease at 37 C for 1 hour in the presence of 1 U/μg DNA in 30 mmol sodium acetate (pH 4.6), 50 mmol NaCl, 1 mmol $ZnSO_4$, and 5% glycerol. SV40 DNA (forms I and II) was included as an internal standard to monitor digestion.

Proteins were recovered from nucleoprotein preparations by precipitation at 4 C in 6 volumes of acetone, with

or without previous extraction in 2.5% TCA or 5% perchloric acid (PCA). After resuspension and denaturation by boiling in 2% sodium dodecyl sulphate (SDS), proteins were separated by electrophoresis in 15% polyacrylamide gels and stained with kenacid blue or silver.[32]

Digestion Kinetics of DNA in Thymocyte Nuclei

Nuclei were prepared by lysis of thymocytes in 10 mmol TRIS pH 7.4, 2 mmol calcium chloride, and 2 mmol PMSF, supplemented with 0.2% Triton X-100 or in this buffer made isotonic by the addition of NaCl to 100 mmol. As internal standards, nuclei of the human lymphoid line Raji, labeled during cell growth by incubation with 1 μCi/ml tritiated thymidine, were added to 1% of the total DNA. The nuclei were washed by centrifugation and resuspended in digestion buffer constituted as above with DNAse I (grade I, Boehringer Corp., Ltd., Lewes, UK) added to 50 or 500 μg/ml. Digestion at 37 C was terminated after various periods between 0 and 60 minutes by the addition of EDTA to 5 mmol. Undigested double-stranded DNA was measured by fluorescence with Hoechst 33258[33] (Hounslow, UK) using excitation and emission wavelengths of 356 and 458 nm, respectively, or by tritium counting of Whatman GF/c filters (Maidstone, UK) on which cells were deposited, washed in saline, and precipitated with ice-cold 5% TCA.

Morphologic Studies on Nuclei

The topography of DNA in apoptotic and morphologically normal thymocytes was studied in protein-depleted, DNA-rich nuclear residues called nucleoids for simplicity, although the method of preparation differs from that used to produce nucleoids of apparently analogous structure from other cell types.[34,35] Nuclei, prepared by isotonic lysis as above, were suspended in large excess volumes of 0.2 M HCl and 0.4 mmol $CaCl_2$ at 4 C for 18 hours. Nucleoids were sedimented by centrifugation (200*g* for 5 minutes) and resuspended swiftly in 100 mmol TRIS, 5 mmol sodium chloride, and 0.5 mmol $CaCl_2$, pH 8.3. A drop of this suspension was mixed on a slide with a drop of acridine orange (10 μg/ml in phosphate-buffered saline) and viewed under a fluorescence microscope.

Nuclear residues depleted of both soluble proteins and DNA—nuclear matrices—were prepared by sequential nuclease digestion and high salt extraction in modifications of previously described methods.[36,37] Nuclei were obtained by isotonic lysis in a buffer consisting of 100 mmol sodium chloride, 1.5 mmol magnesium chloride, and 10 mmol TRIS pH 7.4 (SMT) supplemented with 2 mmol sodium tetrathionate, 0.15% Nonidet P40, 5% sucrose, 1 mmol PMSF, and 250 μg/ml alpha-2-macroglobulin. The nuclei were separated from cytoplasm by centrifugation at 200*g* at 4 C for 10 minutes, resuspended in SMT with 5% sucrose, and incubated either at 37 C for 10 minutes with 500 μg/ml micrococcal nuclease (Sigma) or with 500 μg/ml DNAse I (grade I, BCL) at 20 C for 4 hours. Some matrix preparations were digested with both DNAse I and micrococcal nuclease. The digested nuclei were made up to 2 M NaCl by the addition of a 4 M NaCl solution in gradual increments, and this solution was held at 4 C for 16 hours. Alternatively, some preparations were deproteinized with 0.2 M HCl at 4 C for 16 hours. The matrices were then collected by centrifugation at 200*g* at 4 C for 10 minutes, resuspended in SMT with 5% sucrose, and analyzed for DNA content as above and by SDS–polyacrylamide gel electrophoresis for protein composition. The resulting nuclear matrices were fixed for transmission electron microscopy (TEM) with 3% glutaraldehyde and 0.1 M osmium tetroxide. Thin araldite sections were stained with uranyl acetate and lead citrate and viewed in a Jeol 100S electron microscope (Tokyo, Japan).

Nucleoli of normal and apoptotic thymocytes were studied *in situ* by TEM. In addition to conventionally fixed and stained material, the argyrophilic fibrillar centers were visualized in some preparations by minor modifications of the silver staining method of Hernandez-Verdun et al.[38]

Nuclei from normal thymocytes, isolated by isotonic lysis as described above, were also visualized by TEM before and after incubation at 37 C for up to 10 minutes in micrococcal nuclease (0.25 μ/ml). After incubation directly in isotonic lysis buffer, the nuclei were either fixed and processed for TEM as above or treated with 20 mmol EDTA and 1% SDS for preparation of DNA for agarose gel electrophoresis.

Results

Nuclear Chromatin Is Cleaved at Internucleosomal Sites into Well-organized Chains of Oligonucleosomes

Apoptotic cells and cells of normal morphology, separated on Percoll gradients, were found to contain similar quantities of acid-precipitable DNA: mean values (± S.E.) of 10 estimations were 9.5 (± 0.5) pg for apoptotic cells, 9.2 (± 0.3) pg for untreated cells, and 10.3 (± 0.2) pg for morphologically normal cells after 4 hours of steroid treatment. The physical nature of this DNA was investigated by studying its buoyant density, molecular size, and digestion kinetics.

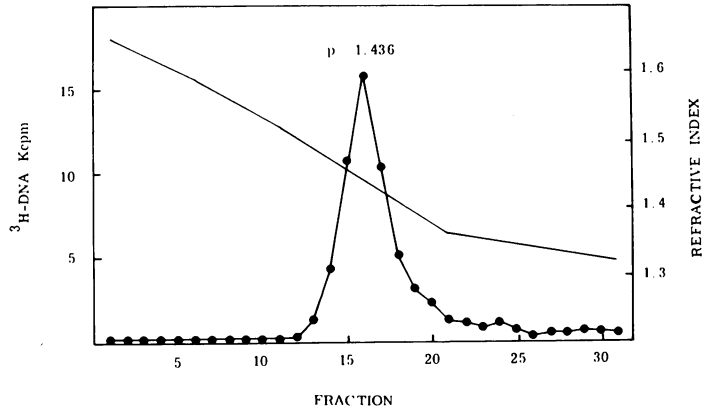


Figure 1. *Tritiated thymidine labeled thymocytes were treated with glucocorticoid to induce apoptosis and the chromatin fragments, recovered after lysis, were fixed in 1% formaldehyde and analyzed by isopyknic centrifugation in cesium chloride. The tritium content (●) of each fraction from the gradient was measured. There is a sharply defined peak with a buoyant density (p) of 1.436 g/ml, indicating that the radiolabeled DNA exists as a nucleoprotein complex. The refractive index (—) of selected fractions from the cesium chloride gradient was measured and used to calculate the buoyant density.*

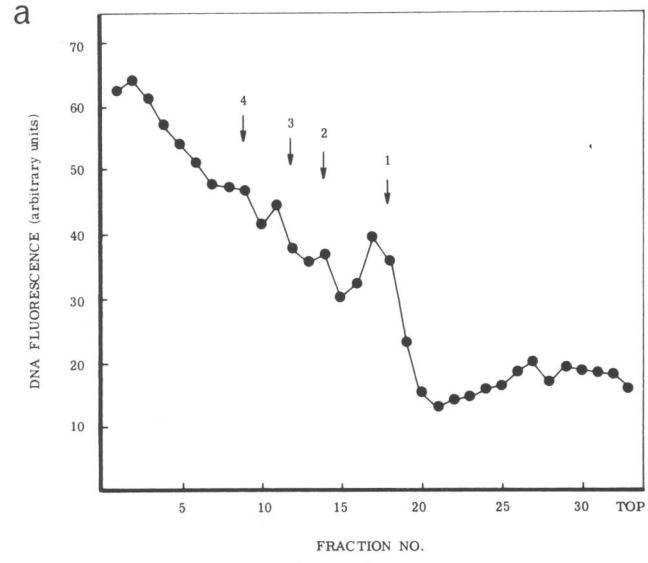
Hypotonic lysates of steroid-treated, 3HTdR-labeled thymocytes were centrifuged at 27,000*g* to procure, in the nonviscous supernatant, the low-molecular-weight DNA fragments from apoptotic cells. After fixation in 1% formaldehyde, these radiolabeled fragments showed a sharply defined buoyant density of 1.436 g/ml in cesium chloride (calculated from the refractive index), indicating that the DNA exists as a nucleoprotein complex (Figure 1). An identical buoyant density was obtained from light micrococcal nuclease digests of chromatin from normal thymocyte nuclei and from Raji cells. High-density material within the cesium chloride gradient, indicative of deproteinized DNA, was not found in preparations from apoptotic cells. Alternatively, the DNA fluorescence of fragments from apoptotic cells was measured after the addition of ethidium bromide to the cesium chloride gradients, and the DNA fragments formed a well-defined peak with a buoyant density of 1.436 ± 0.0037 g/ml (mean ± S.E.; nine estimations).

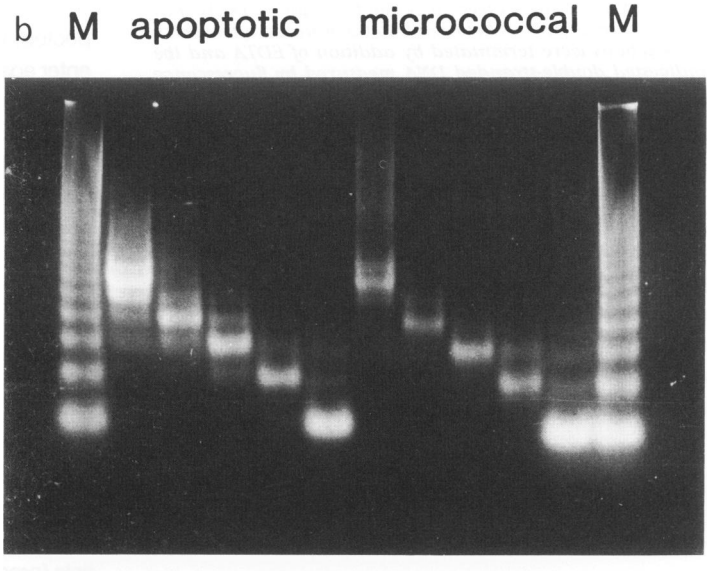
Nucleoprotein fragments from apoptotic cells, prepared as above but without fixation, were studied by velocity sedimentation on sucrose gradients. The sedimentation pattern indicated the presence of particles with stepwise increments in DNA and protein content and integer multiples of a subunit of approximately 185 nucleotide base pairs, identical to authentic oligonucleosomes prepared by micrococcal nuclease digestion of normal thymocyte nuclei (Figure 2A, B). Slowly sedimenting material, indicative of deproteinized DNA, was not observed. This demonstrates that, in apoptosis, chromatin cleavage occurs within internucleosomal linker DNA and that the gross composition of nucleosomes in apoptosis is no different from that of normal cells. In confirmation, polyacrylamide gel electrophoresis demonstrated that the major proteins extracted from the nuclei of apoptotic cells were identical to those of normal cells, which were the four nucleosomal core histones (H3, H2B, H2A, H4) and histone H1 (Figure 2C). This identity does not exclude the possibility of varying degrees of post-translational modification, such as phosphorylation, poly-ADP ribosylation, or methylation.

The digestion kinetics of DNA in nuclei of apoptotic cells were studied to determine whether apoptotic and nonapoptotic cells differed in chromatin packaging and accessibility to nuclease attack. Nuclei were prepared from density-purified apoptotic thymocytes and cells of normal morphology (both untreated and treated with methylprednisolone). The digestion kinetics of DNA were identical in these three types of nuclei when exposed to DNAse I (at concentrations of 50 or 500 μg/ml) under hypotonic and isotonic conditions (Figure 3). To exclude the possibility that endogenous nucleases, or nuclease inhibitors, might be present in differing degrees from these preparations and so influence the digestion kinetics, trace quantities of Raji cell nuclei, labeled with 3HTdR during growth, were included in the incubations. The DNA in these control nuclei was also digested at an identical rate, regardless of whether they were coincubated with nuclei from apoptotic or morphologically normal cells (data not shown).

Chromatin Fragments Are the Products of Double-stranded DNA Cleavage

Oligonucleosomes from apoptotic cells were separated by velocity sedimentation in sucrose gradients. DNA fragments from oligonucleosomes of various lengths (from one to six or eight nucleosomes) were deproteinized and digested with S1 nuclease to detect single-strand nicks or gaps. On subsequent agarose gel electrophoresis, the oligonucleosome-derived DNA had the same mobility, regardless of exposure to S1 nuclease (Figure 4). The ability of the S1 nuclease to detect single-stranded DNA was confirmed by including SV40 DNA as a control in some digestions. At the concentrations used, both form II (nicked circular) and form I (supercoiled) SV40 DNA were completely digested to form III (full-length linear) and





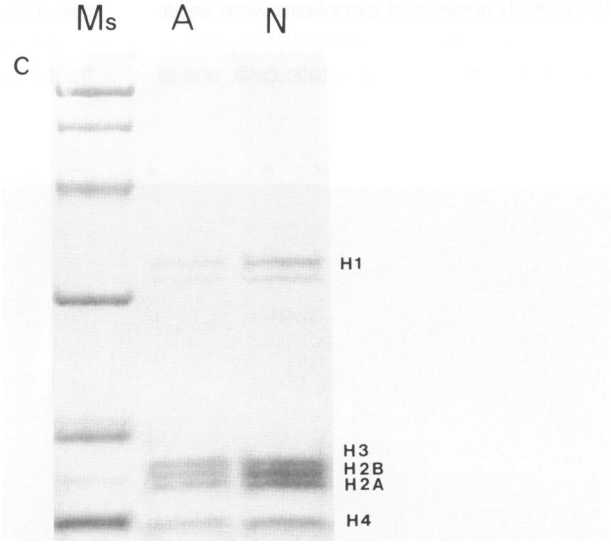


Figure 2. *Chromatin fragments recovered from apoptotic thymocytes were analyzed by velocity sedimentation in 10% to 30% neutral sucrose gradients.* **a:** *The DNA content (●) of each of the gradient fractions was analyzed by fluorescence after addition of ethidium bromide.* **b:** *Fractions containing DNA peaks were further analyzed by 1.8% agarose gel electrophoresis. Particles with stepwise increments in DNA are found in preparations from apoptotic cells (apoptotic). These particles are integer multiples of a 185-bp nucleosomal subunit, varying in length from 1 to 4 nucleosomes and higher orders, identical to authentic oligonucleosomes prepared by micrococcal nuclease digestion of normal thymocyte nuclei (micrococcal). The marker tracks (M) are unfractionated micrococcal nuclease digests of normal thymocyte nuclei.* **c:** *Proteins were recovered from nuclei of isotonically lysed apoptotic* **(A)** *and nonapoptotic* **(N)** *thymocytes and analysed by SDS-polyacrylamide gel electrophoresis. The major proteins are identical in both samples and are the four nucleosomal core histones (H3, H2B, H2A, and H4) and histone H1 (the marker track (Ms) contains electrophoretic size marker proteins of 92, 66, 45, 31, 21 and 14 kilodaltons; the gel was stained with kenacid blue).*

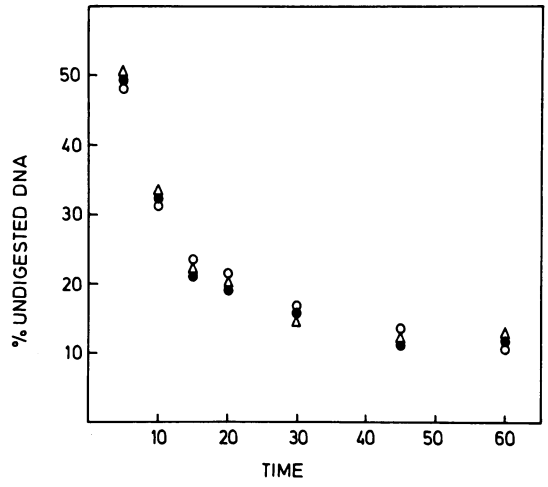


Figure 3. *The digestion kinetics of normal and apoptotic nuclei are shown. Nuclei were prepared from apoptotic (●) and non-apoptotic (○) glucocorticoid-treated, Percoll-purified thymocytes and untreated (△) thymocytes, by isotonic lysis with triton X-100. These nuclei were digested by 50 μg/ml DNAse I at 37C for various periods between 0 to 60 minutes, after which the reactions were terminated by addition of EDTA and the undigested double-stranded DNA measured by fluorescence with Hoechst 33258, and expressed as a percentage of the value at 0 minutes (means of 2 or 3 estimations are shown). Identical digestion kinetics are seen for the three samples.*

smaller fragments (Figure 4). Although form I DNA is covalently closed, its supercoiled configuration creates regions in which single strands are accessible to S1 nuclease.[39] Thus, the absence of further digestion by S1 nuclease of the endogenously cleaved DNA demonstrates that it consisted of double-stranded molecules with no detectable single-strand nicks or gaps.

Two Classes of Oligonucleosome Chain Are Generated

Experiments in which nuclei and cytoplasm were separated after isotonic cell lysis showed that two classes of oligonucleosome chain existed in apoptotic cells, one of which freely dissociated from the nucleus while the other remained bound. Apoptotic cells, purified by centrifugation through Percoll, were lysed under isotonic conditions in the presence of the nonionic detergent NP40 and the protease inhibitor PMSF. Sedimentation at 110*g* produced a pellet consisting of nuclei and a supernatant in which no nuclei or nuclear debris were observed by either phase-contrast microscopy or fluorescence microscopy of acridine orange–stained wet preparations. Low-molecular-weight chromatin was obtained from the nuclear pellet by lysis in hypotonic TRIS–Triton–EDTA buffer and centrifugation at 27,000*g* as before. Of the chromatin DNA in apoptotic cells, 30.4 ± 3.7% failed to sediment at 110 g with the nuclei (mean ± S.E. of 10 estimations), whereas only 4.8 ± 2.0% of the DNA of morphologically normal, steroid-treated cells failed to sediment with the nuclei after similar lysis procedures (five estimations) ($P < 0.01$, Student's *t*-test).

The DNA of morphologically normal cells, as expected, was of very high molecular weight and failed to enter agarose gels. In contrast, the DNA of apoptotic cells (both bound to nuclei and free) displayed the ladder pattern typical of oligonucleosomes. The free DNA consisted mostly of lower-order oligonucleosomes, including mononucleosomes, whereas the DNA cosedimenting with the nucleus had a higher modal molecular weight (Figure 5). Although this bound DNA contained di- and trinucleosomes and higher-order oligonucleosomes, mononucleosomes were observed only in heavily overloaded gel tracks. Normal mononucleosomes could be derived from the nuclear-bound chromatin, however, by further digestion with micrococcal nuclease. Identical results were obtained when the initial cell lysate was centrifuged at up to 3000*g*, confirming that the free and bound oligonucleosomes differed in sedimentation properties because of differential attachment to a large, rapidly sedimenting particle (presumably the nucleus) and not merely because of their intrinsic differences in modal size.

Proteins associated with the bound and free chromatin fractions of the apoptotic cell lysates were studied by

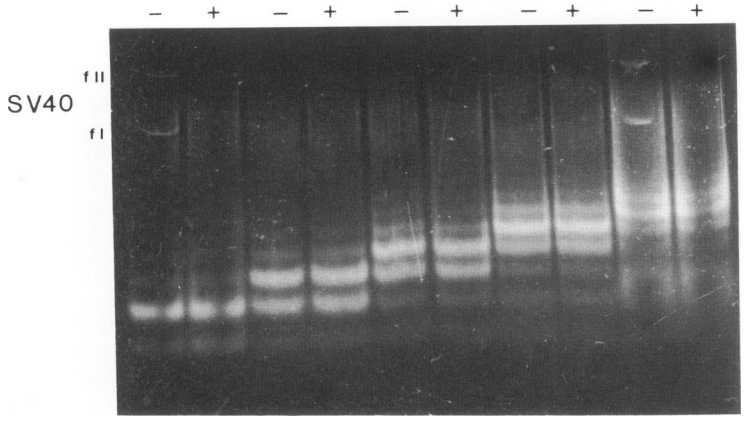


Figure 4. *Agarose gel electrophoresis of deproteinised DNA from sucrose gradient fractionated oligonucleosomes, both before (–) and after (+) S1 nuclease digestion at 37C for 1 hour. SV40 DNA (forms I and II) was included in the mono/di-nucleosome and higher-order oligonucleosome fractions, as positive controls of S1 nuclease activity: there is complete digestion to smaller fragments not visible in the gel. There is no change in the mobility of oligonucleosomes exposed to S1 nuclease, indicating the absence of single-strand nicks.*

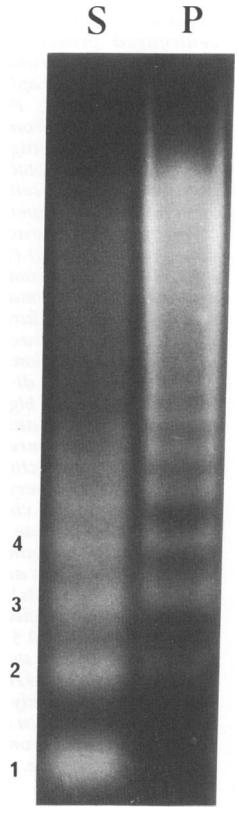


Figure 5. *Agarose gel electrophoresis of DNA recovered from the supernatant(s), containing unattached oligonucleosomes, and the centrifuged pellet (p), containing oligonucleosomes bound to the nuclei of apoptotic cells lysed under isotonic conditions with NP40.*

SDS–polyacrylamide gel electrophoresis. The bound fraction contained abundant histone H1 and the core histones but few HMG proteins, whereas the free fraction was relatively enriched in HMG proteins 1 and 2 and depleted of histone H1 (Figure 6A). In addition to the proteins of the oligonucleosome chains themselves, the free fraction also contained all the freely soluble nuclear proteins, which made uncertain the identification of proteins specifically bound to chromatin. To clarify this, sucrose gradients were used to separate various orders of oligonucleosomes from both the bound and free fractions. This analysis showed clearly that HMG proteins 1 and 2 cosedimented with the free oligonucleosomes (Figure 6B, C). Moreover, the difference in the ratio of H1 to HMG 1 and 2 persisted when oligonucleosomes of similar size were compared, and hence could not be merely the result of the higher proportion of mononucleosomes in the free chromatin fragments. Thus, unbound chromatin fragments are shorter, relatively enriched in HMG 1 and 2, and depleted in H1.

DNA Remains Associated with Residual Nuclear Structures

Nucleoids were prepared by exposing nuclei of apoptotic or morphologically normal cells to 0.2 M HCl. When viewed by fluorescence microscopy after staining with acridine orange (a metachromatic fluorochrome that binds DNA stoichiometrically), the nucleoids prepared from morphologically normal cells had a stippled, bright center and a diffuse peripheral halo, characteristic of long loops of attached DNA. Conversely, the apoptotic nucleoids presented smaller profiles with smooth, sharply defined margins, more intense nuclear staining, and one or more central cavities, consistent with partial loss of DNA after chromatin digestion (Figure 7).

Normal Nuclear Matrix Structures Are Retained

Matrices prepared with DNAse I and high salt, as described in Materials and Methods, contained less than 10% of the original nuclear DNA content and were substantially but not completely depleted of histone (quantitative estimates of bands recovered from gels stained with kenacid blue suggested that at least 80% of the histone was removed). As an alternative, matrices were also produced by a harsher method in which 0.2 M HCl treatment was applied to DNAse I-treated nuclei. This resulted in more complete deproteinization (less than 3% original nuclear protein retained) but less successful removal of DNA (more than 20% retained). These preparations showed ultrastructural features broadly similar to those of the matrices produced by the gentler methods using DNAse I or micrococcal nuclease and high salt. These nuclear matrices displayed a pore complex lamina with an underlying meshwork of fine fibers in which a central fibrillar body, probably representing the residual nucleolus, was observed (Figure 8A to D). Isolated pore complex laminae (empty shells) were observed frequently, as were fragments of fibrillar material, apparently completely dissociated from them and perhaps derived from the nuclear interior. No consistent differences were observed, however, in the preparations obtained from apoptotic and morphologically normal cells. Polyacrylamide gels of the preparations digested with both micrococcal nuclease and DNAse I (with almost complete histone removal) showed many high-molecular-weight proteins with no apparent differences in the matrices of apoptotic and normal origin (Figure 8E).

Nucleolar Constituents Undergo Segregation and Dispersal

The nucleolus was studied in detail in silver-impregnated and conventionally stained preparations of whole cells, both normal and apoptotic. Early in apoptosis (phase 1), the nucleolus condensed into a spherical mass and the

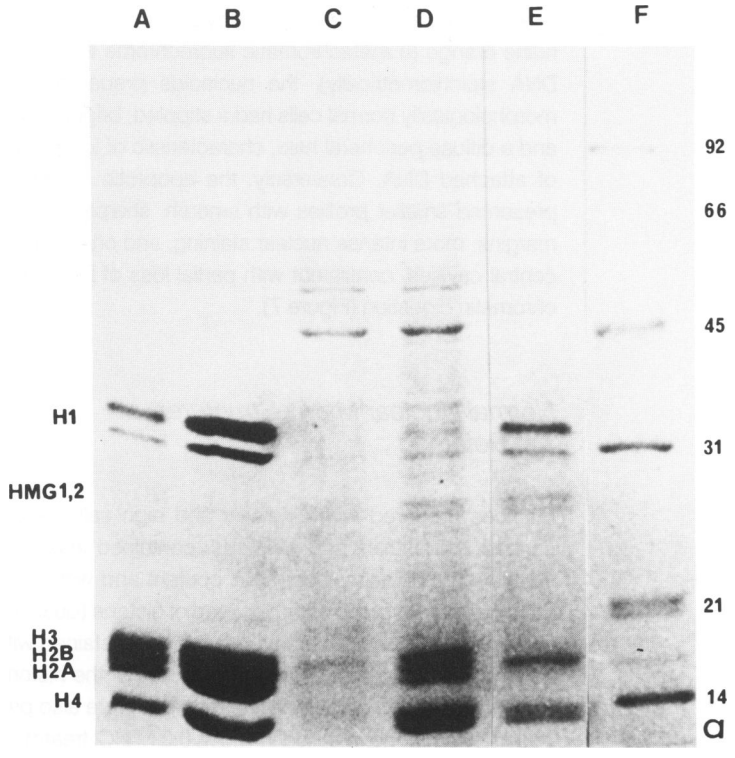


Figure 6. a: *SDS-polyacrylamide gel electrophoresis of proteins recovered from the centrifuged pellets, containing nuclear particle-bound chromatin, of nonapoptotic (track* **A***) and apoptotic (track* **B** *glucocorticoid-treated, Percoll-purified thymocytes, and also from the equivalent supernatants containing free chromatin, of the same nonapoptotic (track* **C***) and apoptotic (track* **D***) cells after isotonic lysis. Proteins from the supernatant of apoptotic thymocytes after extraction in 5% PCA are also shown (track* **E***) (kenacid blue staining). The protein composition of free* **(b)** *and bound* **(c)** *chromatin fragments from apoptotic cells was further analyzed after sucrose gradient fractionation of oligonucleosomes. Fractions containing mononucleosomes (m), di-, and tri- nucleosomes (d/t) and the higher (h)-order oligonucleosomes (4, 5, and 6 nucleosomes in length) were compared, both before (−) and after (+) extraction in 2.5% TCA (although there are very few mononucleosomes in the bound chromatin fragments, the equivalent sucrose gradient fraction is shown) (silver staining). Electrophoretic protein size markers are shown in kilodaltons (tracks F and Ms); histones and HMG proteins were identified both by their solubility in 5% PCA or 2.5% TCA and by comparison with known standards (H1A, H1B, HMG1 and 2, H3 and H4 in track M_H). Free chromatin is relatively enriched in HMG 1 and 2, but depleted in H1A and H1B, compared to bound chromatin, regardless of oligonucleosome size.*

constituents underwent segregation. The argyrophilic fibrillar center was initially surrounded by particles of the osmiophilic dense fibrillar component (DFC) and groups of preribosomal ribonucleoprotein (RNP) granules (Figure 9A to D). As apoptosis progressed, the DFC particles and RNP granules dispersed in the center of the nucleus, whereas the fibrillar center remained intact and was frequently found just internal to the condensed chromatin at the nuclear periphery, separated from it by a narrow osmiolucent furrow (Figure 9E, F).

Micrococcal Nuclease Digestion Reproduces the Nuclear Changes of Apoptosis

In an attempt to reconstruct the events in the apoptotic nucleus, nuclear preparations of normal thymocytes were incubated with micrococcal nuclease, an exogenous nuclease known to cleave both strands of the DNA helix at internucleosomal sites. In order to inactivate proteases, the lysis buffer was supplemented with the protease inhibitor PMSF at relatively high concentration. DNA digestion was monitored by microscopy and agarose gel electrophoresis (Figure 10). After 1 minute of digestion, the nuclear DNA was still of high molecular weight, but the nuclei became perceptibly smaller and the nucleoli more condensed. At 4 minutes, when the DNA existed largely as a polynucleosomal ladder, there was conspicuous peripheral condensation of nuclear chromatin. There was also segregation of nucleolar constituents, which appeared as intact fibrillar centers with adjacent RNP granules and osmiophilic DFC particles, some having undergone dispersal (Figure 10E to G). There was further reduction in nuclear diameter. By 10 minutes, when only lower-order oligonucleosomal fragments of DNA were evident (Figure 10H), the nuclei were reduced to uniformly dense objects about a quarter of their original diameter. Thus, the addition of a purified endonuclease to normal nuclei produces chromatin cleavage and nuclear morphologic changes that parallel those of early apoptosis.

Discussion

An Endonuclease with Double-stranded Preference Is Selectively Activated in Apoptosis

The enzymes presumed to be responsible for chromatin cleavage in apoptosis have not yet been fully characterized, but analysis of the cleavage products can be performed to define some of the properties of the cleavage reaction. We show that chromatin cleavage is affected by endonuclease activity with double-stranded but not sig-

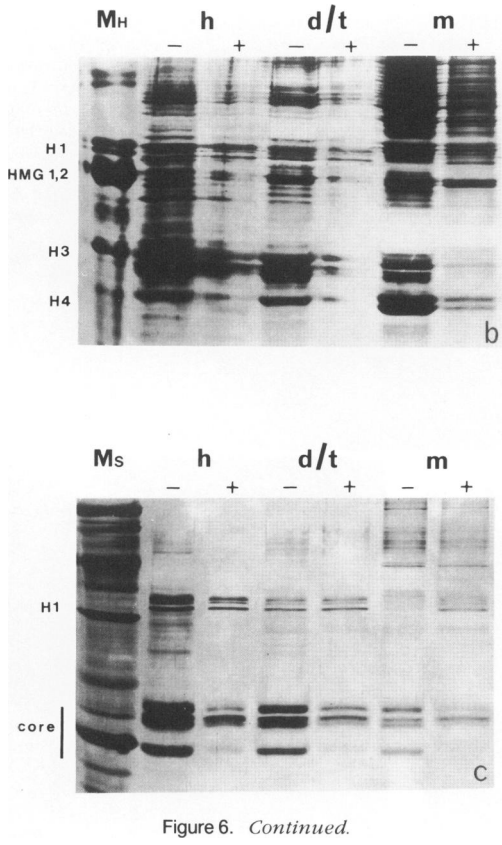


Figure 6. *Continued.*

nificant single-stranded cutting. Additional exonuclease activity has not been formally excluded but appears unlikely on two grounds. First, the modal DNA length associated with mononucleosomes from apoptotic cells is 185 base pairs, and higher oligonucleosomes are exact multiples of this, indicative of substantial conservation of the linker DNA. Second, the endogenous chromatin digestion in apoptosis does not significantly diminish the total acid-precipitable DNA recovered from cells.

The endonuclease activity is apparently not accompanied by indiscriminate proteolytic digestion of chromatin. According to the criteria of nucleoprotein buoyant density, qualitative assessment of the most abundant chromatin-associated proteins, and chromatin susceptibility to exogenous nuclease digestion, apoptotic chromatin is normal. The protein constituents of the nuclear matrix and the ultrastructure of its recovered fragments are substantially the same as those of morphologically normal cells. These interpretations are confirmed by the observation that both the pattern and time course of chromatin cleavage and the morphologic changes in the apoptotic nucleus can be reproduced by brief digestion of normal nuclei in the presence of protease inhibitors with a purified endonuclease (micrococcal nuclease) that cleaves double-stranded DNA. Furthermore, we have previously shown that changes in nuclear morphology, chromatin cleavage, and extractable endonuclease activity follow almost identical time courses after the induction of apoptosis in lymphoid cells by glucocorticoid.[13]

There is evidence that in apoptosis the enzyme responsible for chromatin cleavage may be a neutral endonuclease dependent upon the coincident presence of calcium and magnesium ions and that alteration of the ionic environment in the nucleus may be sufficient to activate this enzyme.[22,28,40,41] Endonucleases with these features are known to be present within the nuclei of many cell types.[41–43] They include the enzyme originally described by Hewish and Burgoyne,[44] which led to the discovery of the nucleosome. It is probable that similar enzymes are responsible for the cleavage of DNA in terminally differentiating lens cells[45] and normoblasts[46] and in some (although perhaps not all) target cells of T-cell–mediated cytotoxicity.[40,47,48] Attempts to purify an enzyme (or enzymes) with these properties from the nuclei of normal thymocytes and certain lymphoid cell lines have already been reported.[49,50]

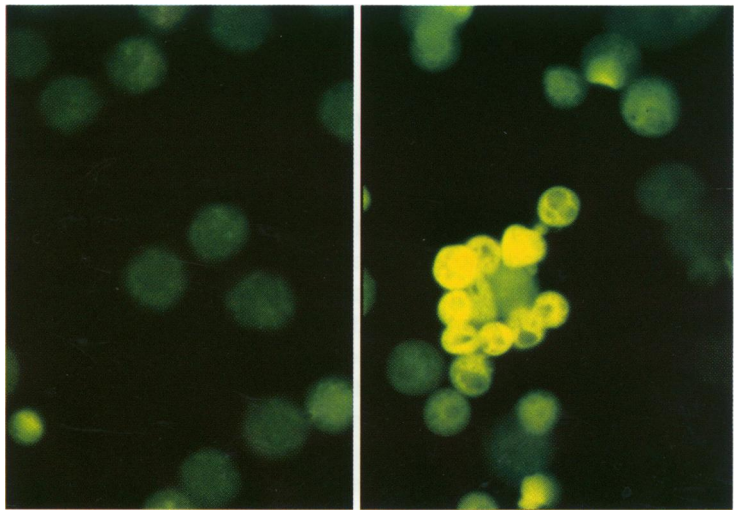


Figure 7. *Nucleoids prepared from* (a, left) *nonapoptotic and* (b, right) *apoptotic thymocytes, after exposure of nuclei to 0.2M HCL. Morphologically normal cell nucleoids have stippled, intensely staining centers and diffuse halos, whereas apoptotic nucleoids are smaller, with well-defined margins, very intense nuclear staining and have central cavities (acridine orange staining; magnification ×1000).*

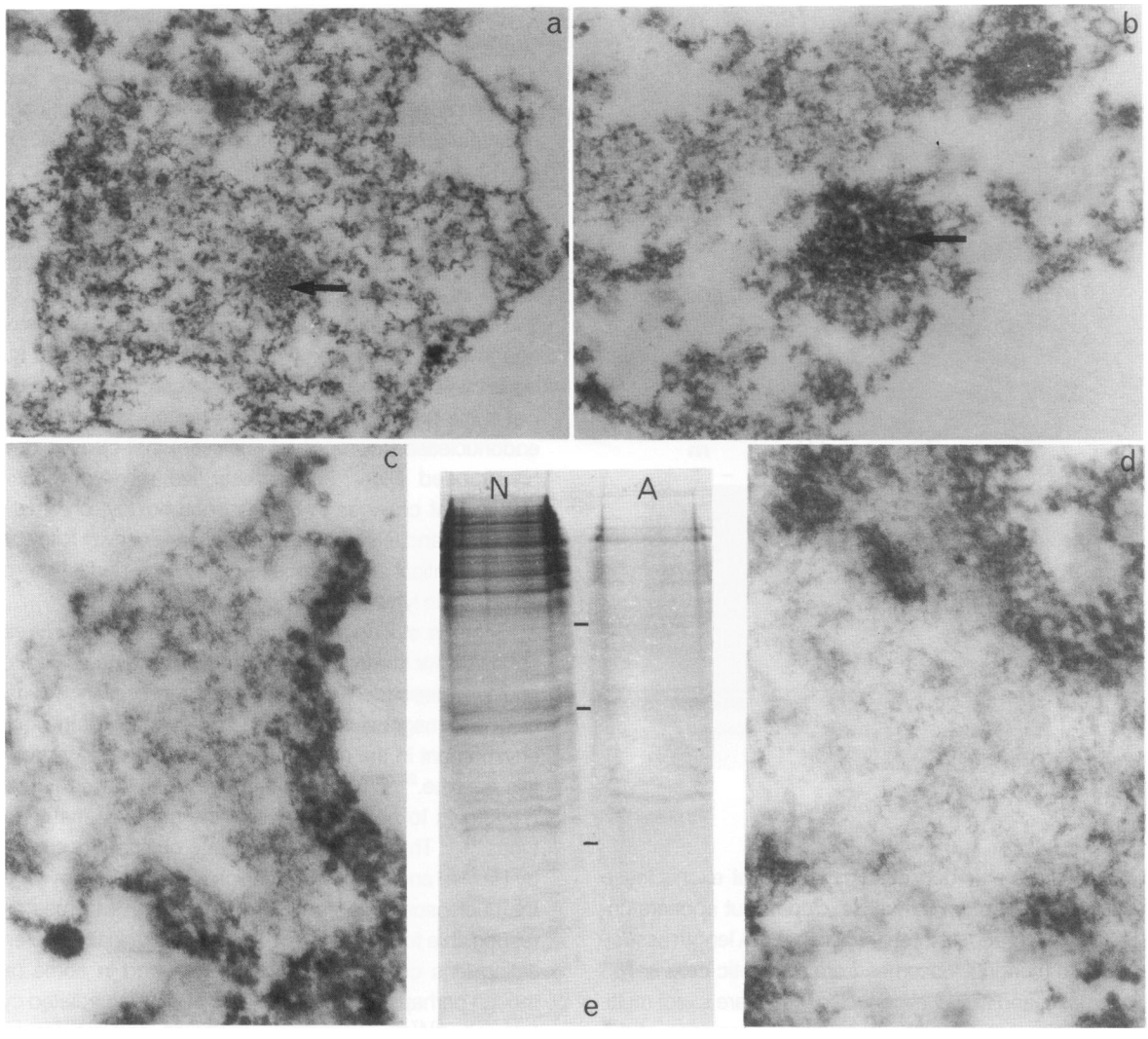


Figure 8. *Matrices from morphologically normal (***a***—mag. ×14000) and apoptotic thymocytes (***b***—mag. ×21000) prepared by micrococcal nuclease digestion and 2-mol NaCl extraction of nuclei. Both show the features of typical nuclear matrices: pore complex laminae, underlying meshworks of fine fibres and central fibrillar bodies (arrows), regarded as the residual nucleoli. Matrix preparations of DNAse I digested, 2-mol NaCl extracted nuclei, from morphologically normal (***c***—mag. ×40,000) and apoptotic (***d***—mag. ×40,000) thymocytes both display a closely similiar fine fiber meshwork and pore complex lamina (uranyl acetate and lead citrate staining). SDS-polyacrylamide gel electrophoresis (***e***) of matrices digested with both DNAse I and micrococcal nuclease and extracted with 2-mol NaCl, prepared from morphologically normal (**N**), and apoptotic (**A**) thymocytes (with almost complete histone removal) shows an almost identical pattern of many high-molecular-weight proteins (silver staining—the horizontal bars between the two tracks represent molecular size markers of 92, 66, and 45 kd).*

The Pattern of DNA Digestion Is Influenced by Pre-existing Chromatin Organization

Chromatin packaging occurs at multiple levels: DNA is wrapped twice around histone octomers (two each of H3, H2A, H2B, and H4) to form nucleosomes separated by linker DNA in a 110 A–diameter beads-on-a-string structure.[51] This may be further assembled into a 300 A filament believed to be formed by winding of the chain of nucleosomes into a solenoid containing six nucleosomes per turn, with the linker DNA and histone H1 on the inside.[52,53] Interactions between H1 molecules possibly mediate formation of the solenoid, although other models have been suggested.[52,53] Large loops of such compacted DNA (up to 100 Kb) are attached at their bases to the protein matrix of the nucleus in association with topoisomerase II[54,55] with further stacking of these loops.[56] We demonstrate that in apoptosis, as a result of activation of an endogenous endonuclease, chromatin is cleaved into oligonucleosome chains with different properties, presumably reflecting the configuration of the chromatin from which they derive.

Chromatin that remains attached to the nuclear structure is incompletely digested and contains long oligo-

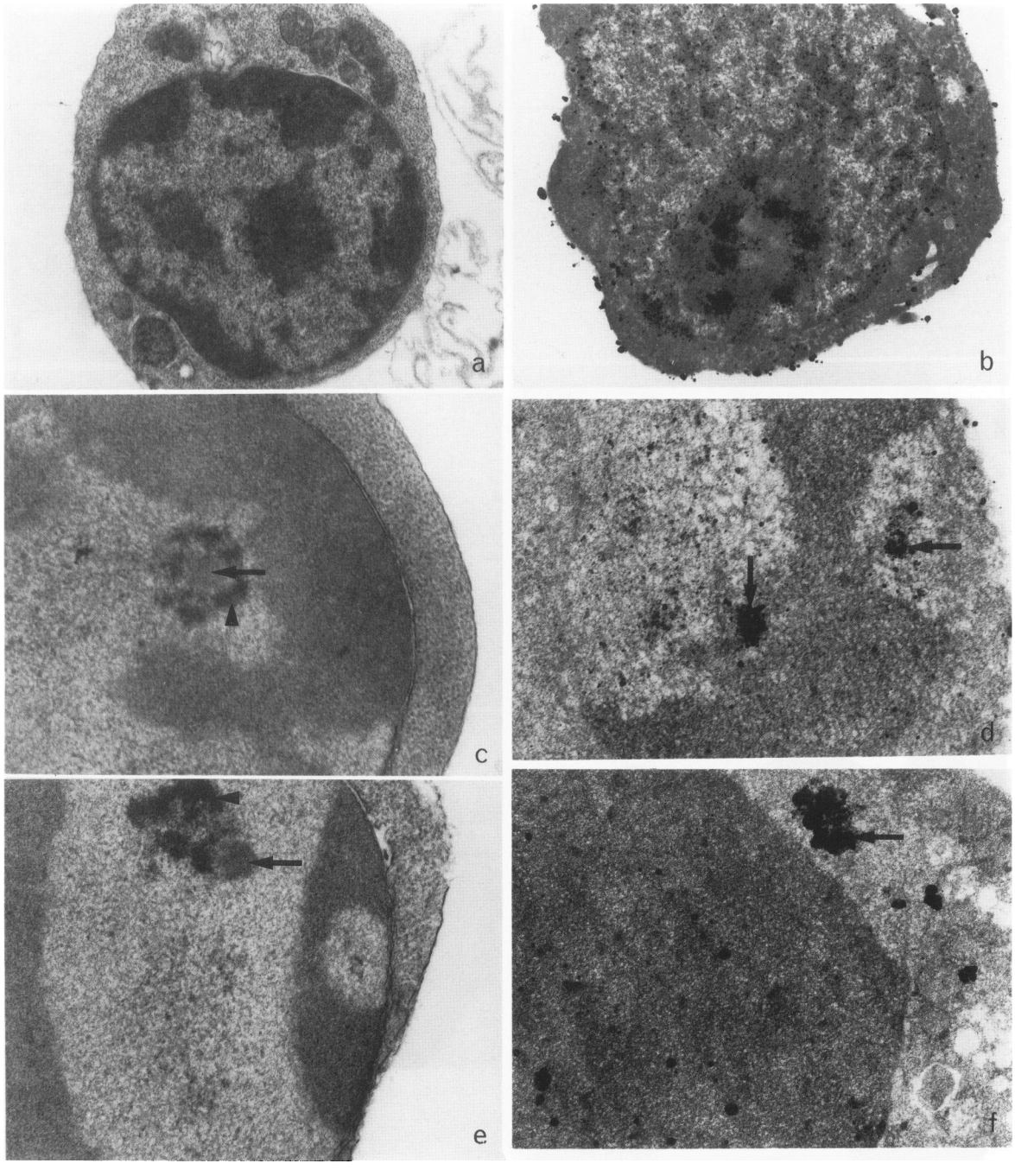
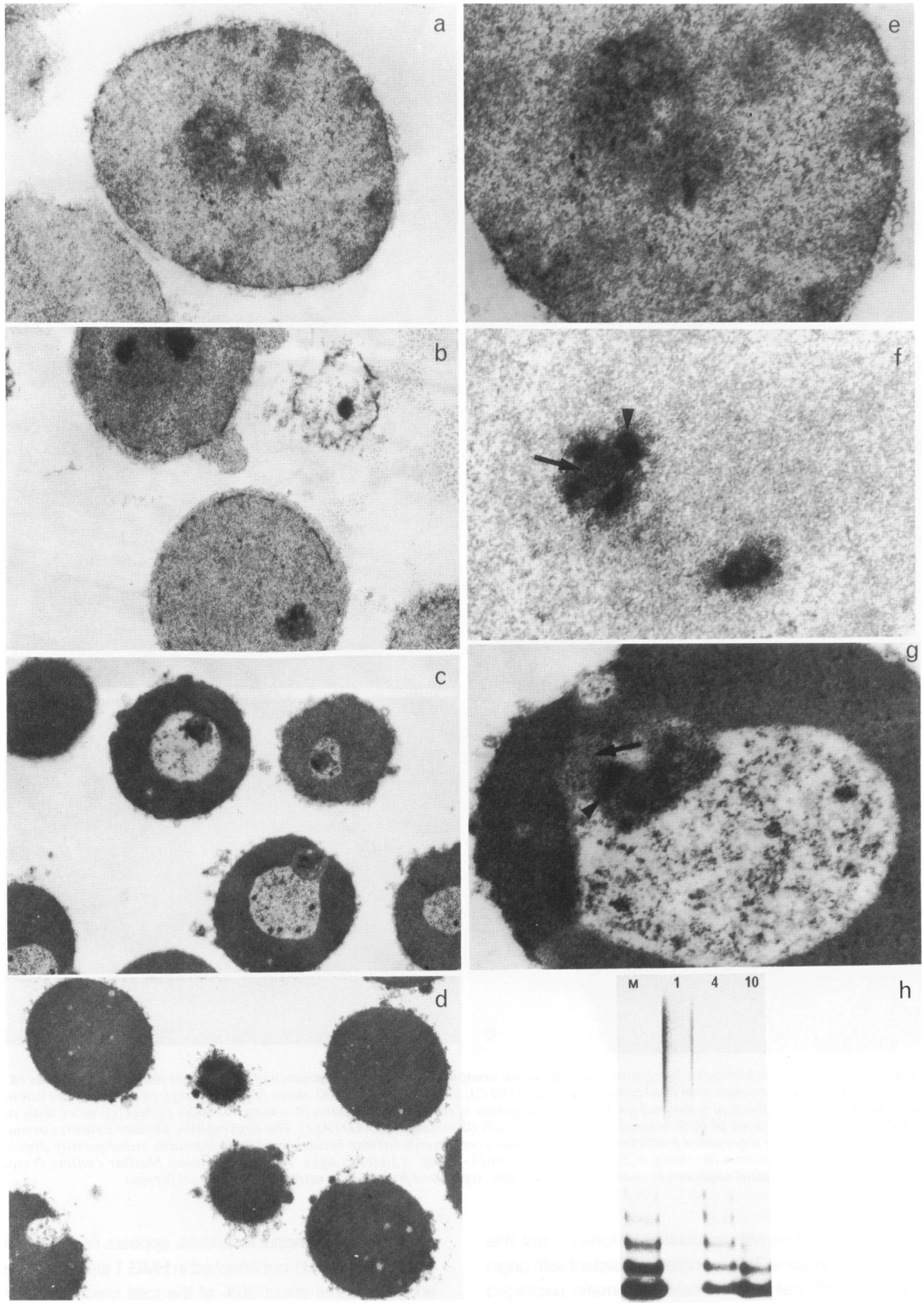


Figure 9. *Nucleolar morphologic changes in early apoptosis compared with the appearance of nucleoli in normal thymocytes (***a***—mag. ×10,000, uranyl acetate and lead citrate staining (UA/LC); and* **b***—mag. ×8000, silver (Ag) staining). In apoptosis the nucleolus condenses into a spherical mass and undergoes segregation of its constituent parts (***c***—mag. ×25000, (UA/LC)); more than one fibrillar center may be seen in both normal and apoptotic cells (***d***—mag. ×30,000 (Ag)). The argyrophilic fibrillar centers (arrows) are conserved and the segregated particles of dense fibrillar components (arrow heads), and RNP granules, subsequently disperse in the center of the nucleus (***e***—mag. ×25,000 (UA/LC); and* **f***—mag. ×30,000 (Ag)). The silver-stained fibrillar centers remain intact and are often found adjacent to condensed chromatin, separated from it by a narrow osmiolucent furrow.*

nucleosomal chains with associated histone H1 but little HMG 1 or 2 proteins. This is entirely consistent with origin from transcriptionally inactive heterochromatin, packaged as the 300 A solenoid.[52] In contrast, the chromatin that is not attached to the nuclear structure contains a high proportion of mononucleosomes, appears relatively deficient in histone H1 but enriched in HMG 1 and 2 proteins, and represents about 30% of the total chromatin DNA. HMG 1 and 2 proteins are thought to replace H1 at the junction of linker and nucleosome-wrapped DNA, thus in-


a
e
b
f
c
g
d
h
M
1
4
10

creasing the accessibility of chromatin to polymerases and other enzymes.[57-59] They have been shown to stimulate transcription by RNA polymerase II, perhaps by facilitating the formation of active initiation complexes.[60,61] Hence, the free chromatin in apoptotic cells has many features suggesting origin from chromatin in a transcriptionally active configuration. In comparison with the 300 A solenoid, such chromatin would be expected to be more accessible to digestion by soluble nucleases in the nucleoplasm.[57,62] Further evidence that endogenous endonucleases preferentially digest transcriptionally active genes comes from studies of hen oviduct nuclei. Here the ovalbumin gene, activated by hormone stimulation, is preferentially digested by endogenous nucleases, relative to both the transcriptionally inactive globin gene and the ovalbumin gene in unstimulated cells.[63]

Chromatin fibers of normal nuclei are anchored to the nuclear matrix or scaffold,[56,64,65] apparently through binding of certain consensus DNA sequences.[55] The number and spacing of such anchorages may vary with the pattern of gene expression according to differentiation.[55,66] Anchorage to the nuclear matrix defines supercoiled DNA loops that become evident on removal of histones from normal nuclei[34,35] and are visualized as the diffuse halo around the nucleus in preparations (nucleoids) treated with high salt solution or dilute acid. The partial loss of this DNA halo in similar preparations made from apoptotic nuclei is exactly as would be predicted following multiple cleavage events between anchorage sites. A similar pattern of partial loss of chromatin fibers surrounding the nuclear structure was visualized by electron microscopy in irradiated thymocytes undergoing apoptosis.[67] Unlike chromatin in 110 A nucleosomal chains, condensed chromatin may be maintained as aggregates of 300 A filaments, even after cleavage of DNA at internucleosomal sites, through protein–protein interactions between adjacent coils of the solenoid.[68] This affords a simple explanation for the retention of as much as 70% of DNA attached to the nuclear structure, despite being the target of multiple DNA cleavage events. The proportion of DNA in the normal nucleus in the condensed form is estimated as at least 70%.[62] Matrix preparations of both early apoptotic and normal thymocyte nuclei are similar in protein composition and contain indistinguishable pore complex laminae, fine fiber meshworks, and residual nucleolar bodies. Thus, in apoptosis there is selective activation of an endonuclease without apparent degradation of the nuclear matrix, but the pre-existing organization of chromatin loops attached to the matrix[56] influences the pattern of DNA digestion. The mechanisms by which apoptotic nuclei undergo budding and separation into several chromatin-containing bodies in phase 2 apoptosis are not understood.

Cleavage of rDNA May Explain the Nucleolar Morphologic Changes

Although nucleolar morphologic changes have long been recognized as characteristic of apoptosis,[1,15,69] this study is the first attempt to analyze them in detail. In particular, we show that the changes must be interpretable on the basis of nuclease activation alone, because they can be reproduced by micrococcal nuclease digestion of intact nuclei in the presence of protease inhibitors. We show that the fibrillar center, including the argyrophilic protein nucleolin, retains its normal topography even in nuclei with extensively condensed chromatin, but that the DFC is digested into many particles, and both they and the preribosomal RNP granules disperse at an early stage. This pattern is similar to that seen in nucleoli following treatment with actinomycin D.[70,71] This transcriptional inhibitor causes nucleolar segregation: transcription complexes in the DFC undergo dissociation, and RNP granules are released into adjacent nucleoplasm, but the fibrillar center is conserved. Cells in which rRNA synthesis has ceased, such as avian eythrocytes, also show intact fibrillar centers without surrounding RNP granules.[72] In apoptosis, the morphologic changes can be explained by cleavage of transcriptionally active ribosomal genes (rDNA) within the dense fibrillar components,[73,74] thus forming multiple, variably sized DFC particles that, along with RNP granules, undergo segregation, become detached from the surface of the fibrillar center, and subsequently disperse. The protein-rich fibrillar center itself remains intact. Its frequent location close to the peripheral condensed chromatin perhaps suggests continuity of the protected transcriptionally inactive internal rDNA with adjacent chromosomal DNA.[74]

Nuclease Activation May Aid Disposal of Dead Cells and Protect Against Gene Transfer

In conclusion, we have presented evidence that endogenous endonuclease activity is a major component of the

Figure 10. *Micrococcal nuclease digestion of normal thymocyte nuclei produces nuclear morphologic changes parallel to those of early apoptosis. Compared with nuclei* (**a**—*mag. ×6000*) *and nucleoli* (**e**—*mag. ×10000*) *at time zero, after 1 minute of digestion, nuclei become smaller* (**b**—*mag. ×6000*) *and nucleoli condense* (**f**—*mag. ×20000*). *At 4 minutes there is peripheral condensation of chromatin* (**c**—*mag. ×6000*) *and segregation of nucleoli into intact fibrillar centers (arrows) and adjacent particles of dense fibrillar component (arrow heads) and RNP granules* (**g**—*mag. ×20,000*), *which undergo dispersal. At 10 minutes, nuclei appear as uniformly dense, chromatin-containing spheres* (**d**—*mag. ×6000*) *(uranyl acetate and lead citrate staining). Agarose gel electrophoresis of extracted DNA* (**h**) *shows a smear of high-molecular-weight DNA after 1 minute of digestion (1), cleavage to a polynucleosomal ladder by 4 minutes (4) and to lower-order oligonucleosomes by 10 minutes (10) (the marker track* (**M**) *is a micrococcal nuclease digest of normal thymocyte chromatin producing an oligonucleosomal ladder).*

process of apoptosis, being responsible for the chromatin condensation and nucleolar segregation and dispersal, which are among the most distinctive morphologic features. The question arises as to the functional significance of endonuclease activity early in intrinsically programmed cell death. One result of chromatin cleavage and subsequent condensation is a dramatic reduction in nuclear bulk, permitting budding and separation of the nuclear material into several small packages of condensed chromatin. This may aid the formation of small apoptotic bodies of a size suitable for phagocytosis by neighboring viable cells or macrophages. Furthermore, we have shown that transcriptionally active chromatin is apparently preferentially cleaved, presumably because of its accessible configuration. Double-stranded cleavage of intranuclear supercoiled DNA has been shown to cause immediate and profound loss of transcriptional activity.[75] It is thus probable that activation of endogenous endonuclease produces this effect in apoptotic cells. We have previously recorded radiolabeled uridine incorporation of apoptotic cells as less than 9% of normal.[12] Because apoptotic cells usually undergo phagocytosis by their neighbors within tissues (which may not be specialized macrophages), the cleavage of DNA to low-molecular-weight material may serve a protective function limiting the probability of transfer of genes in a potentially active state from dying cells to the nuclei of their viable neighbors.

References

1. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
2. Wyllie AH: Cell death: A new classification separating apoptosis from necrosis, Cell Death in Biology and Pathology. Edited by ID Bowen, RA Lockshin. London, New York, Chapman and Hall Press, 1981, pp. 9–34
3. Searle J, Kerr JFR, Bishop CJ: Necrosis and apoptosis: Distinct modes of cell death with fundamentally different significance. Pathol Annu 1982, 17(2):229–259
4. Hinchliffe JR: Cell death in embryogenesis, Cell Death in Biology and Pathology. Edited by ID Bowen, RA Lockshin. London, New York, Chapman and Hall Press, 1981, pp 35–78
5. Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JT: Antibodies to CD3/T-Cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature 1989, 337:181–184
6. Shi Y, Sahai BM, Green DR: Cyclosporin A inhibits activation-induced cell death in T-cell hybridomas and thymocytes. Nature 1989, 339:625–626
7. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of ageing neutrophils in inflammation: Programmed cell death in the neutrophil leads to its recognition by macrophages. J Clin Invest 1989, 83:865–875
8. Duke RC, Cohen JJ: IL-2 addiction: Withdrawal of growth factor activates a suicide program in dependent T cells. Lymphokine Res 1986, 5:289–299
9. Kerr JFR, Searle J: Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Arch [B] 1973, 13:87–102
10. Rotello RJ, Hocker MB, Gerschenson LE: Biochemical evidence for programmed cell death in rabbit uterine epithelium. Am J Pathol 1989, 134:491–495
11. Sandow BA, West NB, Norman RL, Brenner RM: Hormone control of apoptosis in hamster uterine luminal epithelium. Am J Anat 1979, 156:15–36
12. Wyllie AH, Morris RG: Hormone-induced cell death: Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. Am J Pathol 1982, 109:78–87
13. Wyllie AH, Morris RG, Arends MJ, Watt AE: Nuclease activation in programmed cell death, coordinated Regulation of Gene Expression. Edited by RM Clayton, DES Truman. New York, Plenum Press, 1986, pp 33–41
14. Russel JH. Internal disintegration model of cytotoxic lymphocyte-mediated cytoxicity. Biol Rev 1981, 56:153–197
15. Wyllie AH: Cell death. Int Rev Cytol 1987, 17(Suppl):755–785
16. Duvall E, Wyllie AH: Death and the cell. Immunol Today 1986, 7:115–119
17. Yamada T, Ohyama H: Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). Int J Radiat Biol 1988, 53:65–75
18. Skalka M, Matyasova J, Cejkova M: DNA in chromatin of irradiated lymphoid tissues degrades in vivo into regular fragments. FEBS Lett 1976, 72:271–274
19. Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980, 284:555–556
20. Wyllie AH: Apoptosis. ISI Atlas Science: Immunology 1988, 1:192–196
21. McConkey DJ, Nicotera P, Hartzell P, Bolloma G, Wyllie AH, Orrenius S: Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch Biochem Biophys 1989, 269:365–370
22. McConkey DJ, Hartzell P, Nicotera P, Orrenius S: Calcium activated DNA fragmentation kills immature thymocytes. FASEB Journal 1989, 3:1843–1849
23. Barnes DM: Cells without growth factors commit suicide. Science 1988, 242:1510–1511
24. Buttyan R, Zakeri Z, Lockshin R, Wolgemuth D: Cascade induction of c-fos, c-myc and heat shock 70K transcripts during regression of the rat ventral prostate gland. Mol Endocrinol 1988, 2:650–657
25. Wyllie AH, Rose KA, Morris RG, Steel CM, Foster E, Spandidos DA: Rodent fibroblast tumors expressing human myc and ras genes: Growth, metastasis and endogenous oncogene expression. Br J Cancer 1987, 56:251–259
26. Wyllie AH, Morris RG, Smith AL, Dunlop D: Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 1984, 142:67–77

27. Wyllie AH: The biology of cell death in tumours. Anticancer Res 1985, 5:131–136
28. Cohen JJ, Duke RC: Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 1984, 132:38–42
29. Burton K: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 1956, 62:315–323
30. Sharp PA, Sugden B, Sambroo KJ: Detection of two restriction endonuclease activities in Haemophilus parainfluenzae using analytical agarose. Biochemistry 1973, 12:3055–3063
31. Jackson V, Chalkley R: Separation of newly synthesized nucleohistone by equilibrium centrifugation in caesium chloride. Biochemistry 1974, 13:3952–3956
32. Oakley BR, Kirsch DR, Morris NR: A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 1980, 105:361–363
33. Labarca C, Paigen K: A simple, rapid and sensitive DNA assay procedure. Anal Biochem 1980, 102:344–352
34. Cook PR, Brazell IA, Jost E: Characterization of nuclear structures containing superhelical DNA. J Cell Sci 1976, 22: 303–324
35. Vogelstein B, Pardoll DM, Coffey DS: Supercoiled loops and eucaryotic DNA replication. Cell 1980, 22:79–85
36. Bouvier D, Hubert J, Bouteille M: The nuclear shell in HeLa cell nuclei: Whole-mounted electron microscopy of the dissociated and isolated nuclear periphery. J Ultrastruct Res 1980, 73:288–298
37. Kaufmann SH, Coffey DS, Shaper DS: Consideration in the isolation of rat liver nuclear matrix, nuclear envelope and pore complex lamina. Exp Cell Res 1981, 132:105–123
38. Hernandez-Verdun D, Hubert J, Bourgeouis C, Bouteille M: Identification ultrastructurale de l'organisateur nucleolaire par la technique a l'argent. C R Acad Sci [III] 1978, 287: 1421–1423
39. Beard P, Morrow JF, Berg P: Cleavage of circular, superhelical Simian Virus 40 DNA to a linear duplex by S1 nuclease. J Virol 1973, 12:1303–1313
40. Allbritton N, Verret CR, Wolley RC, Eisen HN: Calcium ion concentrations and DNA fragmentation in target cell destruction by murine cloned cytotoxic T lymphocytes. J Exp Med 1988, 167:514–527
41. Ishida R, Akiyoshi H, Takahoshi T: Isolation and purification of calcium and magnesium dependent endonuclease from rat liver nuclei. Biochem Biophys Res Commun 1974, 56: 703–710
42. Nakamura M, Sakaki Y, Watanabe N, Takagi Y: Purification and characterization of the Ca^{2+} plus Mg^{2+} dependent endodeoxyribonuclease from calf thymus chromatin. J Biochem 1981, 89:143–152
43. Liu LF, Liu CC, Alberts BM: Type II topoisomerases: Enzymes that can unknot a topologically knotted DNA molecule via a reversible double-strand break. Cell 1980, 19:697–707
44. Hewish DR, Burgoyne LA: Chromatin substructure. The digestion of chromatin at regularly spaced sites by a nuclear deoxyribonuclease. Biochem Biophys Res Commun 1973, 52:504–510
45. Appleby DW, Modak SP: DNA degradation in terminally differentiating lens fiber cells from chick embryos. Proc Natl Acad Sci USA 1977, 74:5579–5583
46. Williamson R: Properties of rapidly labelled deoxyribonucleic fragments from cytoplasm of primary cultures of embryonic mouse liver. J Mol Biol 1970, 51:157–168
47. Martz E, Howell DM: CTL: Virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. Immunol Today 1989, 10:79–86
48. Howell DM, Martz E: The degree of CTL-induced DNA solubilization is not determined by the human vs mouse origin of the target cell. J Immunol 1987, 138:3695–3698
49. Wyllie AH, Morris RG, Watt AE: Terminally differentiated and dying lymphoid cells contain nuclear endonuclease potentially responsible for chromatin changes of apoptosis. J Pathol 1986, 148:94A
50. Dykes TA, McCall C, Weiner L, Duke R, Cohen JJ: Purification and characterization of the endogenous endonuclease involved in programmed cell death of developing thymocytes. Clin Res 1987, 35:139A
51. Richmond TJ, Finch JT, Rushton B, Rhodes D, Klug A: Structure of the nucleosome core particle at 7A resolution. Nature 1984, 311:532–537
52. Finch JT, Klug A: Solenoidal model for superstructure in chromatin. Proc Natl Acad Sci USA 1976, 73:1897–1901
53. Pederson DS, Thoma F, Simpson RT: Core particle, fiber and transcriptionally active chromatin structure. Ann Rev Cell Biol 1986, 2:117–147
54. Newport JW, Forbes DJ: The nucleus: Structure, function and dynamics. Annu Rev Biochem 1987, 56:535–565
55. Gasser SM, Laemmli UK: A glimpse at chromosomal order. Trends in Genet 1987, 3:16–22
56. Nelson WG, Pienta KJ, Barrack ER, Coffey DS: The role of the nuclear matrix in the organisation and function of DNA. Annu Rev Biophys Biophys Chem 1986, 15:457–475
57. Jackson JB, Pollock JM, Rill RL: Chromatin fractionation procedure that yields nucleosomes containing near-stoichiometric amounts of high mobility group non-histone chromosomal proteins. Biochemistry 1979, 18:3739–3748
58. Levy BW, Dixon GH: Partial purification of transcriptionally active nucleosomes from trout testis cells. Nucleic Acids Res 1978, 5:4155–4163
59. Jackson JB, Rill RL: Circular dichroism, thermal denaturation, and deoxyribonuclease I digestion studies of nucleosomes highly enriched in high mobility group proteins HMG 1 and HMG 2. Biochemistry 1981, 20:1042–1046
60. Tremethick DJ, Molloy PL: High mobility group proteins 1 and 2 stimulate transcription in vitro by RNA polymerases II and III. J Biol Chem 1986, 261:6986–6992
61. Tremethick DJ, Molloy PL: Effects of high mobility group proteins 1 and 2 on initiation and elongation of specific transcription by RNA polymerase II in vitro. Nucleic Acids Res 1988, 16:11107–11123
62. Gottesfeld JM: Chromatin fractionation and the properties of transcriptionally active regions of chromatin, Chromatin Structure and Function: Levels of Organization and Cell Function. Edited by CA Nicolini. New York, London, Plenum Press, 1978, pp 541–560

63. Vanderbilt JN, Bloom KS, Anderson JN: Endogenous nuclease: Properties and effects on transcribed genes in chromatin. J Biol Chem 1982, 257:13009–13017
64. Shaper JH, Pardoll DM, Kaufmann SH, Barrack ER, Vogelstein B, Coffey DS: The relationship of the nuclear matrix to cellular structure and function. Adv Enzyme Regul 1979, 17:213–248
65. Long BH, Huang C-Y, Pogo AO: Isolation and characterisation of the nuclear matrix in Friend erythroleukaemic cells: Chromatin and hnRNA interactions with the nuclear matrix. Cell 1979, 18:1079–1090
66. Buongiorno-Nardelli M, Gioacchina M, Carri MT, Marilley M: A relationship between replicon sites and supercoiled loop domains in the eukaryotic genome. Nature 1982, 298:100–102
67. Yamada T, Ohyama H, Kinjo Y, Watanabe M: Evidence for the internucleosomal breakage of chromatin in rat thymocytes irradiated in vitro. Radiat Res 1981, 85:544–553
68. Warant RW, Kim SH: Alpha-helix–double helix interaction shown in the structure of a protamine-transfer RNA complex and a nucleoprotamine model. Nature 1978, 271:130–135
69. Wyllie AH, Kerr JFR, Currie AR: Cell death: The significance of apoptosis. Int Rev Cytol 1980, 68:251–356
70. Jordan EG, McGovern JH: The quantitative relationship of the fibrillar centres and other nucleolar components to changes in growth conditions, serum deprivation and low doses of actinomycin D in cultured diploid human fibroblasts. (Strain MRC-5). J Cell Sci 1981, 52:373–389
71. Simard R: The nucleus: Action of chemical and physical agents. Int Rev Cytol 1970, 28:169–211
72. Laval M, Hernandez-Verdun D, Bouteille M: Remnant nucleolar structures and residual RNA synthesis in chick erythrocytes. Exp Cell Res 1981, 132:157–167
73. Goessens G, Lepoint A: The nucleolus-organizing regions (NOR's): Recent data and hypotheses. Biol Cellulaire 1979, 35:211–220
74. Jordan G: Transcription at the heart of the nucleolus. Nature 1987, 329:489–490
75. Harland RM, Weintraub H, McKnight SL: Transcription of DNA injected into Xenopus oocytes is influenced by template topology. Nature 1983, 302:38–43

Acknowledgments

The authors wish to thank Irene Evans and R. Hogg for their technical assistance, Catherine Clarke and P.S. Aggutter, in the Department of Biological Sciences, Napier College, for assistance with some of the matrix preparations, and Seona Macintosh for preparation of the manuscript. The electrophoretic protein standards comprising histones H1, H3, and H4, and also HMG proteins 1 and 2, were the gift of Dr. K. Gooderham.