Journal of Cellular Biochemistry 74:99–110 (1999)

# Biochemical and Morphological Changes in the Nuclear Matrix Prepared From Apoptotic HL-60 Cells: Effect of Different Stabilizing Procedures

**Alberto M. Martelli,* Roberta Bortul, Renato Bareggi, Vittorio Grill, Paola Narducci, and Marina Zweyer**

Dipartimento di Morfologia Umana Normale, Università di Trieste, I-34138 Trieste, Italy

**Abstract** Apoptotic cell death is characterized by deep morphological changes that take place in the nucleus. It is unclear whether modifications also occur in the nuclear matrix, a mainly proteinaceous structure that conceivably acts as a nuclear framework. We have investigated whether biochemical and morphological alterations of the nuclear matrix prepared from apoptotic HL-60 cells were dependent on the manipulations to which isolated nuclei were subjected before DNase I digestion and 2 M NaCl extraction. Our results showed that the stabilizing procedures employed to preserve the inner fibrogranular network and nucleolar remnants of the matrix (i.e., a 37°C incubation; exposure to sodium tetrathionate at 4°C; exposure to sodium tetrathionate at 37°C) had no effect on the protein recovery of apoptotic nuclear matrices, which was always approximately two- to fivefold less than in control matrices. Moreover, one- and two-dimensional gel analysis of nuclear matrix proteins showed that, in apoptotic samples, striking quantitative changes were present, as compared with controls. Once again, these changes were seen irrespective of the stabilizing procedures employed. Also, transmission electron microscope analysis showed similar morphological alterations in all types of apoptotic nuclear matrices. By contrast, the immunofluorescent distribution of the 240-kDa NuMA protein seen in apoptotic samples was more sensitive to the stabilizing treatments. Our results indicate that the biochemical and morphological changes of the apoptotic nuclear matrix are largely independent of the isolation protocols and strengthen the contention that destruction of the nuclear matrix network is one of the key events leading to apoptotic nuclear destruction. J. Cell. Biochem. 74:99–110, 1999. 

**Key words:** apoptosis; nuclear matrix; stabilization; protein composition; ultrastructural analysis; immunocytochemistry

Apoptosis is a form of active cell death that is involved in normal and pathological biological processes, such as embryonic development, tissue renewal and regression, differentiation of immunocompetent cells, and tumor development [Bellamy et al., 1995; Jacobson et al., 1997; Leist and Nicotera, 1997]. Apoptosis can be induced in cells by a multitude of noxious agents, including radiation, hyperthermia, and a plethora of toxins [Hale et al., 1996; Vaux and Strasser, 1996].

Because its implications extend from toxin-induced cell death through immunological control to developmental biology and cancer, apoptotic cell death is a widely studied phenomenon. After commitment of cells to apoptosis, characteristic morphological changes are observed in the execution phase of cell death. These alterations include cytoplasmic compaction; cell shrinkage and separation; condensation of chromatin that marginates toward the inner nuclear membrane, forming cap-shaped, compact areas; and, ultimately, fragmentation of residual nuclear structures into apoptotic bodies that are phagocytosed by neighboring cells and macrophages [Earnshaw, 1995; Kroemer et al., 1995; Hale et al., 1996; Vaux and Strasser, 1996; Martelli et al., 1997].

The biochemical mechanisms of apoptotic execution are still under intensive study, with particular attention focused on the role of proteases and nucleases [Hale et al., 1996; Vaux and Strasser, 1996; Villa et al., 1997]. A great deal is now known about the identity and targets of the proteolytic enzymes, such as caspases, serine proteases, calpains, and granzymes that appear to be the driving force behind the apop-

Grant sponsor: Fondi Associazione Italiana per la Ricerca sul Cancro(A.I.R.C.); Grant sponsor: Università di Trieste.

*Correspondence to: A.M. Martelli, Dipartimento di Morfologia Umana Normale, Università di Trieste, via Manzoni 16, I-34138 Trieste, Italy. E-mail:martelli@univ.trieste.it

Received 9 December 1998; Accepted 21 January 1999

totic pathway [Cohen, 1997; Thornberry et al., 1997]. A hallmark feature of apoptosis was the observation that nuclear DNA extracted from apoptotic cells was often degraded by endogenous nucleases into large and finally nucleosomal-size fragments [e.g., Collins et al., 1997]. The large DNA fragments likely correspond to the 50- to 100-kb chromatin loops in which the genome is organized during the interphase [Lagarkova et al., 1995]. These loops interact with the nuclear matrix through specialized AT-rich DNA regions called scaffold-associated regions (SARs) or matrix-associated regions (MARs) [Boulikas, 1995; Bode et al., 1996].

The nuclear matrix was originally defined as the insoluble and salt-resistant proteinaceous nuclear scaffold isolated from interphase cells after digestion with nucleases and extraction with high-ionic-strength buffers [Berezney et al., 1995; Martelli et al., 1996]. It is thought to constitute the three-dimensional filamentous protein network that maintains the domain organization of the nucleus [Berezney et al., 1995; Martelli et al., 1996]. Consisting of the outer lamina, the inner fibrogranular network, and residual nucleoli, the matrix plays a role in diverse structure-bound processes such as anchoring of DNA loops, DNA replication and transcription, RNA processing and transport, steroid hormone action, and signal transduction, as well as a plethora of other functions [Berezney et al., 1995; Nickerson et al., 1995].

Given the dramatic nuclear changes that occur during apoptosis, and considering the fact that several of the proteolyzed proteins belong to the nuclear matrix [e.g., Martelli et al., 1997, and references therein], the matrix would be expected to exhibit marked changes during the apoptotic execution. Nevertheless, the data reported by Arends et al. [1990] excluded any differences in both the morphology and the protein composition of nuclear matrix prepared from dexamethasone-treated thymocytes, as compared with controls. Subsequently, however, several studies, mainly based on the use of antibodies in immunocytochemical investigations, have suggested that the nuclear matrix might be disassembled during the apoptotic process [Miller et al., 1993; Zweyer et al., 1995, 1997; Tinnemans et al., 1995; Weaver et al., 1996; Casiano et al., 1996; Sodja et al., 1998]. A very critical issue in the field of nuclear matrix is that various protocols have been employed to isolate it. It is well known that even apparently minor changes in the isolation methods have marked consequences on the morphology and biochemical composition of the final matrix structure [Nickerson et al., 1995; Martelli et al., 1996]. We have very recently demonstrated that when the nuclear matrix is prepared from apoptotic HL-60 by a very gentle procedure, both biochemical and morphological changes are detactable, as compared with controls [Martelli et al., 1999]. As our method differs substantially from the protocol employed by Arends et al. [1990], we decided to investigate to what an extent the isolation protocols may influence the biochemical composition and the ultrastructural aspects of matrices prepared from apoptotic HL-60 cells. In this paper, we show that, irrespective of the manipulations to which isolated nuclei were subjected in vitro before extraction procedures, both biochemical and ultrastructural changes could be detected in the nuclear matrix prepared from apoptotic cells. By contrast, the isolation protocols influenced the distribution in apoptotic nuclear matrices of the 240-kDa NuMA protein.

## MATERIALS AND METHODS

### Cell Culture and Induction of Apoptosis

HL-60 human promyelocytic leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). To induce apoptosis, cells were synchronized at the $G_1$/S border with 1 μg/ml aphidicolin for 15 h. They were then washed three times in RPMI-1640 to remove aphidicolin. Aliquots of cells at this stage were used as control cells. For induction of apoptosis, cells were exposed for 3 h to 0.1 μg/ml of the DNA topoisomerase I inhibitor, camptothecin (Sigma Chemical Co., St. Louis, MO), according to Del Bino et al. [1991]. In some cases, exponentially growing cells were incubated with 68 μM etoposide (Sigma) for 3 h according to Martins et al. [1997]. Flow cytometric analysis revealed that both of these treatments caused about 65–70% of cells to undergo apoptosis, whereas in control samples, $<$1% of cells were apoptotic [data not shown; also Martelli et al., 1999].

### Preparation of Nuclear Matrix from HL-60 Cells

To isolate nuclei, the procedure by Martelli et al. [1991] was followed. Briefly, cells were washed once in phosphate-buffered saline (PBS) (without $Ca^{2+}$ and $Mg^{2+}$) and resuspended to

$1.5 \times 10^7$/ml in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml each of aprotinin and leupeptin (TM-2 buffer, temperature = 10°C). After 5 min at 0°C, Triton X-100 was added to 0.5% (w/v), and cells were sheared by one passage through a 22-gauge needle fitted to a 30-ml plastic syringe. Nuclei were sedimented at 400*g* for 6 min, washed once in TM-2 buffer, and resuspended to 2 mg DNA/ml in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$ plus protease inhibitors as above (STM-5 buffer). They were incubated for 45 min under the following conditions: (1) 0°C in STM-5 buffer; (2) 37°C in STM-5 buffer; (3) 0°C in STM-5 buffer containing 2 mM sodium tetrathionate (NaTT); and (4) 37°C in STM-5 buffer containing 2 mM NaTT. Nuclei were then digested for 60 min at 0°C with 50 U DNase I/mg DNA (Sigma). An equal volume of 4 M NaCl in 10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF (LM buffer) was then added, followed by 8 vol of 2 M NaCl in LM buffer. Structures were sedimented at 1,500*g* for 10 min, washed once in LM buffer, and employed as the nuclear matrix fraction.

### Protein Recovery

Assays were performed as described by Bradford [1976].

### SDS-PAGE and Immunoblotting Analysis

Nuclear matrix protein (from $3 \times 10^7$ cells) was dissolved in electrophoresis sample buffer [Laemmli, 1970] and the proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting analysis, protein separated on 12% SDS-PAGE was transferred to nitrocellulose sheet, using a semidry blotting apparatus (Hoefer/Pharmacia Biotech, Uppsala, Sweden). Sheets were saturated in PBS containing 5% normal goat serum (NGS) and 4% bovine serum albumin (BSA) for 60 min at 37°C (blocking buffer), and then incubated overnight at 4°C in blocking buffer containing a monoclonal antibody to protein B23 (a kind gift from Dr. R.L. Ochs, Scripps Research Institute, La Jolla, CA) diluted 1:2,000. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-mouse IgG, diluted 1:3,000 in PBS-Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence method (Boehringer, Mannheim, Germany).

### Two-Dimensional Gel Electrophoresis

Nuclear matrix protein (from $5 \times 10^7$ cells) was resuspended in 400 µl of lysis buffer [O'Farrell et al., 1975] and incubated for 3 h at room temperature. Insoluble material was removed by centrifugation at 10,000*g* for 5 min, and the supernatant was layered on the first dimension gel. Nonequilibrium pH gradient gel electrophoresis (NEPHGE) was carried out as reported by O'Farrell et al. [1977] in 9.2 M urea, 2% Nonidet P-40 (NP-40), 4% polyacrylamide, 2% ampholytes (Bio-Lyte pH 3–10, Bio-Rad Laboratories, Milan, Italy). First-dimension gels (10 cm long with a diameter of 3 mm) were run for 16 h at 300 V (constant). Second-dimension gels were 8% SDS-PAGE. Gels were stained with Coomassie Blue R-250. The pH gradient was calibrated using carbamylated glyceraldehyde-3-phosphate dehydrogenase standards (Pharmacia Biotech). Gels representative of three separate preparations are shown.

### Transmission Electron Microscopy Analysis

Structures were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 35 min. They were embedded in Araldite and observed with a Jeol 100S transmission electron microscope (TEM).

### Immunofluorescent Staining

The procedure was carried out essentially as described by Neri et al. [1997]. Nuclear matrix structures in PBS were plated onto 0.1% poly-L-lysine-coated glass slides; adhesion was allowed to proceed for 30 min at room temperature. Samples were fixed in freshly prepared 4 % paraformaldehyde in PBS for 30 min at room temperature. After several washes with PBS, nonspecific binding of antibodies was blocked by 30-min incubation at 37°C with PBS, 2% BSA, 5% NGS. Slides were then incubated for 3 h at 37°C with a monoclonal antibody to the N-terminal domain of NuMA protein (Transduction Laboratories, Lexington, KY) in PBS, 2% BSA, 5% NGS. Slides were then washed three times in PBS and reacted with fluorescein isothiocyanate-conjugated anti-mouse IgM (Sigma), diluted 1:100 in PBS, 2% BSA, 5% NGS for 1 h at 37°C. Slides were observed and

photographed using a Zeiss Axiophot epifluorescence microscope.

### Statistical Analysis

Data are the mean from three different experiments and are expressed as mean ±SD. Significant differences ($P < 0.01$) in a Student's paired *t*-test are indicated in the table by an asterisk.

## RESULTS

### Protein Recovery and Composition of Nuclear Matrix Prepared from Control and Apoptotic HL-60 Cells

Apoptotic as well as nonapoptotic cells were investigated for their nuclear matrix obtained by the well-characterized protocol of Martelli et al. [1991]. As shown in Table I, protein assays showed that the nuclear matrix prepared from control HL-60 cells retained about 2.5-fold more protein than the matrix obtained from apoptotic cells, irrespective of the treatment to which isolated nuclei were subjected before DNase I digestion and salt extraction. All differences between control and apoptotic matrices were found to be significant ($P < 0.01$) in a Student's paired *t*-test.

SDS-PAGE analysis of the polypeptides present in the nuclear matrix revealed the existence of mainly quantitative differences between control and apoptotic cells (Fig. 1). Once again, this was largely independent of the manipulations to which nuclei were subjected. It is worth mentioning, however, that we observed a striking quantitative difference in the recovery of a protein migrating at approximately 38 kDa. Coomassie Blue staining showed that it was much more represented in matrices stabilized by NaTT than in those stabilized by moderate heat (37°C) or in those exposed to both NaTT and heat. The molecular weight of this polypeptide reminded us of protein B23/numatrin, which is known to be highly enriched in the nuclear matrix prepared from nuclei stabilized by NaTT [Fields et al., 1986]. By immunoblotting analysis with a monoclonal antibody recognizing protein B23, we demonstrated that this polypeptide was indeed more abundant if the matrix was stabilized by NaTT alone than by other treatments (Fig. 2). However, the same

**TABLE I. Percentage of Nuclear Protein Recovered in Nuclear Matrix Prepared from Control and Apoptotic HL-60 Cells**[a]

| Condition | Inducer | Control | Apoptotic |
|---|---|---|---|
| 0°C nuclear matrix (a) | Campothecin | 7.4 ± 1.9 | 2.8 ± 0.8* |
| 37°C nuclear matrix (b) | Camptothecin | 20.6 ± 2.8 | 6.1 ± 1.8* |
| 0°C NaTT nuclear matrix (c) | Camptothecin | 23.8 ± 3.1 | 9.1 ± 2.1* |
| 37°C NaTT nuclear matrix (d) | Camptothecin | 25.6 ± 2.9 | 10.0 ± 2.2* |
| 37°C NaTT nuclear matrix (d) | Etoposide | 27.5 ± 3.5 | 10.5 ± 2.5* |

[a]The results are the mean of three different preparations ±SD.
*Significant differences ($P < 0.01$) in a Student's paired *t*-test.

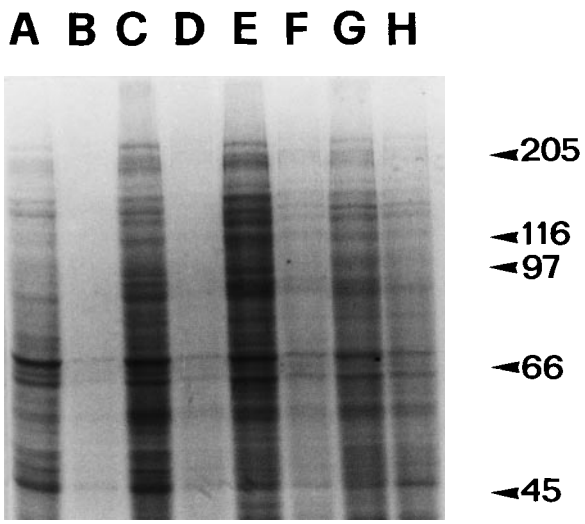



**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of nuclear matrix protein from control (lanes A,C,E,G) and camptothecin-treated (lanes B,D,F,H) HL-60 cells. Nuclear matrix prepared from nuclei incubated **(A,B)** at 0°C; **(C,D)** at 37°C; **(E,F)** at 0°C in the presence of 2 mM NaTT; and **(G,H)** at 37°C in the presence of 2 mM NaTT. The position of molecular-weight markers is indicated on the right.

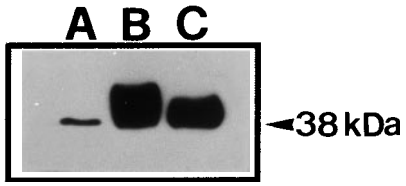



**Fig. 2.** Immunoblotting analysis for protein B23 in isolated nuclear matrices prepared from control HL-60 cells. Nuclear matrix prepared from nuclei incubated at 37°C **(lane A)**; at 0°C in the presence of 2 mM NaTT **(lane B)**; and at 37°C in the presence of 2 mM NaTT **(lane C)**.

behavior was also seen in apoptotic samples (data not shown).

Because the polypeptide composition of the nuclear matrix is exceedingly complicated [Berezney et al., 1995], we analyzed the proteins of the nuclear matrix prepared from control and apoptotic samples, by two-dimensional gel electrophoresis (Fig. 3). It is evident that, with this technique, primarily quantitative differences were seen between apoptotic and control cells, confirming the results of the one-dimensional analysis shown in Figure 1.

Obviously, each stabilizing treatment produced its own distinctive polypeptide pattern, in agreement with our own previous observations [Martelli et al., 1995], but quantitative differences between normal and apoptotic samples were always readily identifiable. To rule out the possibility that changes in the protein composition of the nuclear matrix prepared from apoptotic cells were attributable exclusively to the inducer camptothecin, we also employed etoposide, another powerful pro-apoptotic chemical for the HL-60 cell line. As

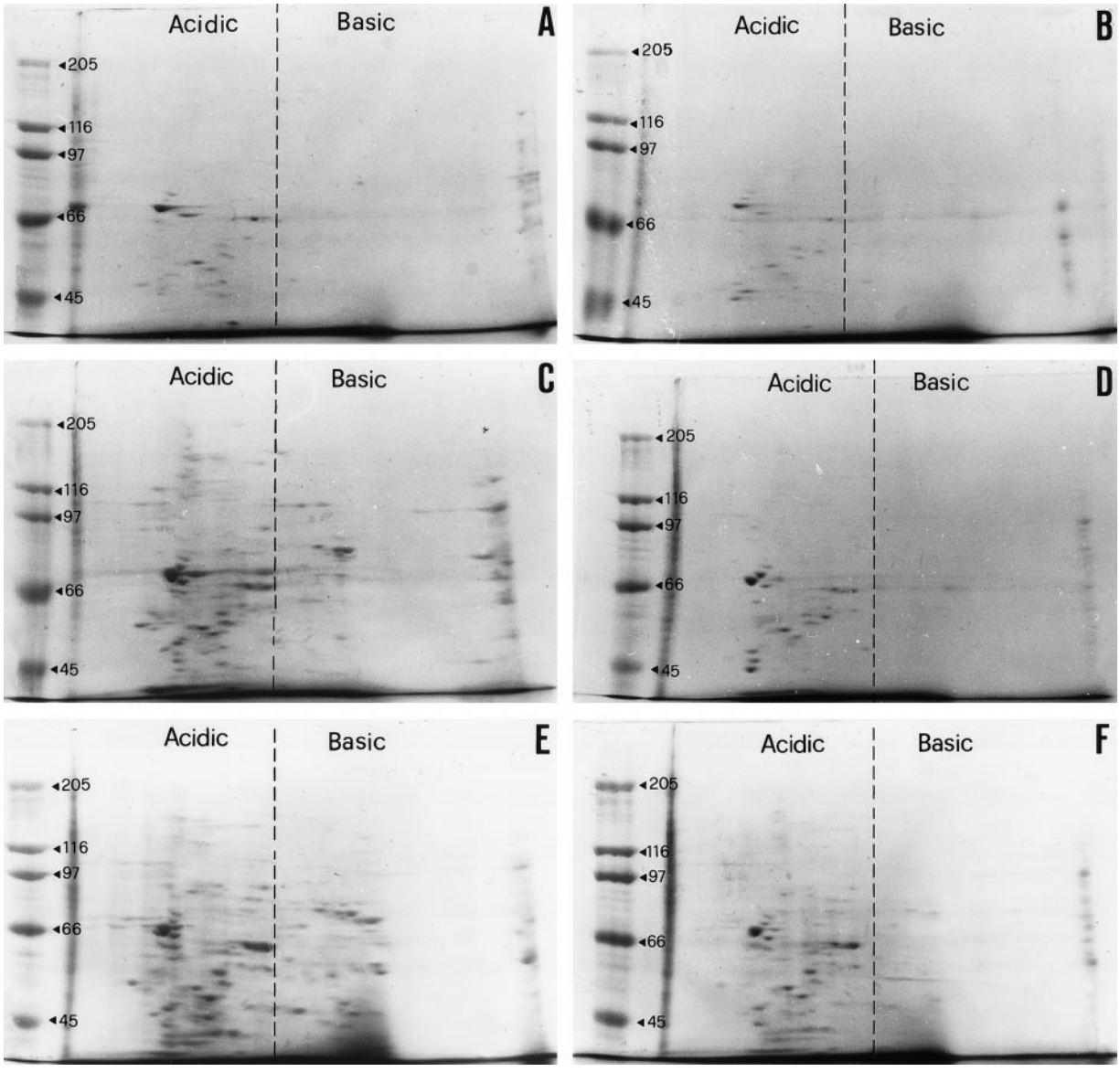



**Fig. 3.** Two-dimensional gel electrophoresis analysis of protein recovered in the nuclear matrix prepared from control **(A,C,E)** and camptothecin-treated **(B,D,F)** HL-60 cells. Nuclear matrix prepared from nuclei (A,B) incubated at 37°C; (C,D) at 0°C in the presence of 2 mM NaTT; and (E,F) at 37°C in the presence of 2 mM NaTT. Migration in the first dimension was from left to right. Dashed lines, pH 7.0. Molecular-weight markers are indicated on the left.

shown in Figure 4, striking quantitative changes in the protein composition of the apoptotic cell nuclear matrix were seen by two-dimensional gel electrophoresis in samples treated for 3 h with 68 μM etoposide. In this case, nuclei had been stabilized by incubation with NaTT at 37°C (condition d, see Materials and Methods).

### TEM Analysis

We next investigated the morphology of our nuclear matrix preparations, by TEM analysis. As expected, nucleoskeletal structures obtained from control cells exhibited a peripheral lamina, an inner fibrogranular network, and nucleolar remnants, independent of the stabilizing procedure (Fig. 5A–C). There were some differences, however. For example, the amount of the fibrogranular network was higher in sample stabilized by 37°C incubation alone (Fig. 5A) or 37°C plus NaTT (Fig. 5C) than in those exposed to NaTT alone (Fig. 5B).

In preparations obtained from apoptotic cells, besides nuclear matrices, which looked equal to control matrices (Fig. 6A,E), and which conceivably derived from cells that were not yet apoptotic, we detected matrices with the nucleolar remnants characterized by a central cavity (Fig. 6C). Moreover, in all the samples, we saw nucleolar remnants surrounded by residues of the fibrogranular network (Fig. 6B,D,F). Some of these nucleolar remnants also exhibited a central cavity (Fig. 6D,F). Moreover, the periphery of the nucleolar remnants, as well as the peripheral ring surrounding the internal electron-transparent areas, showed very electron-

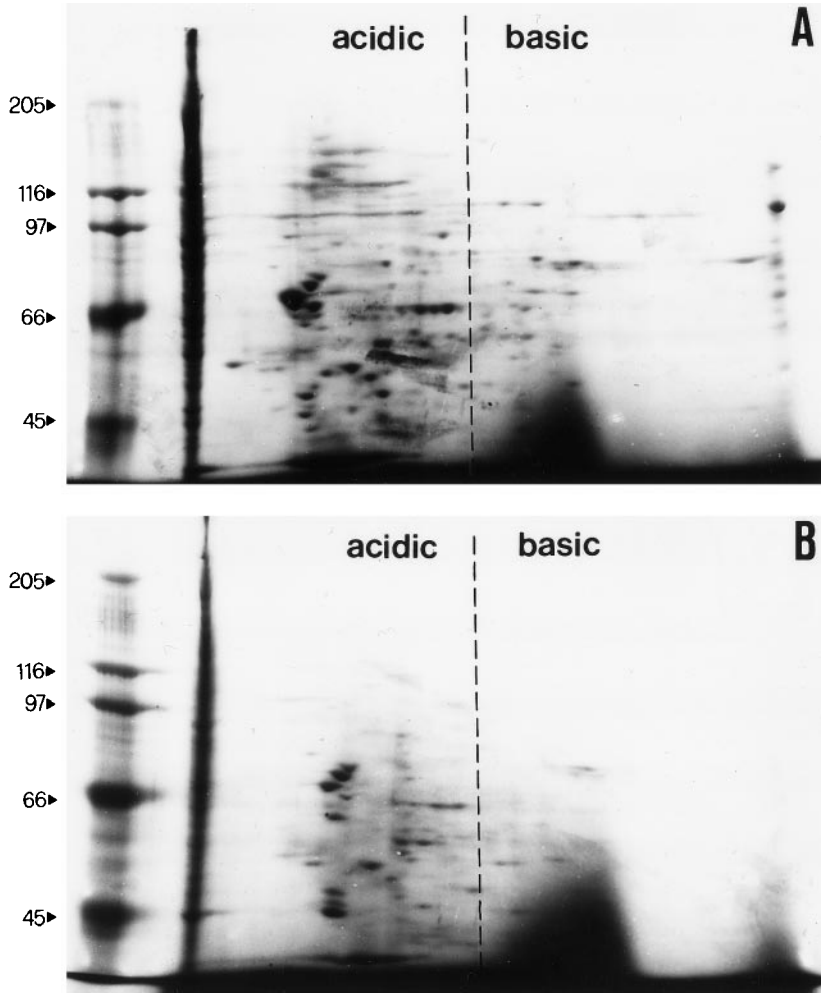
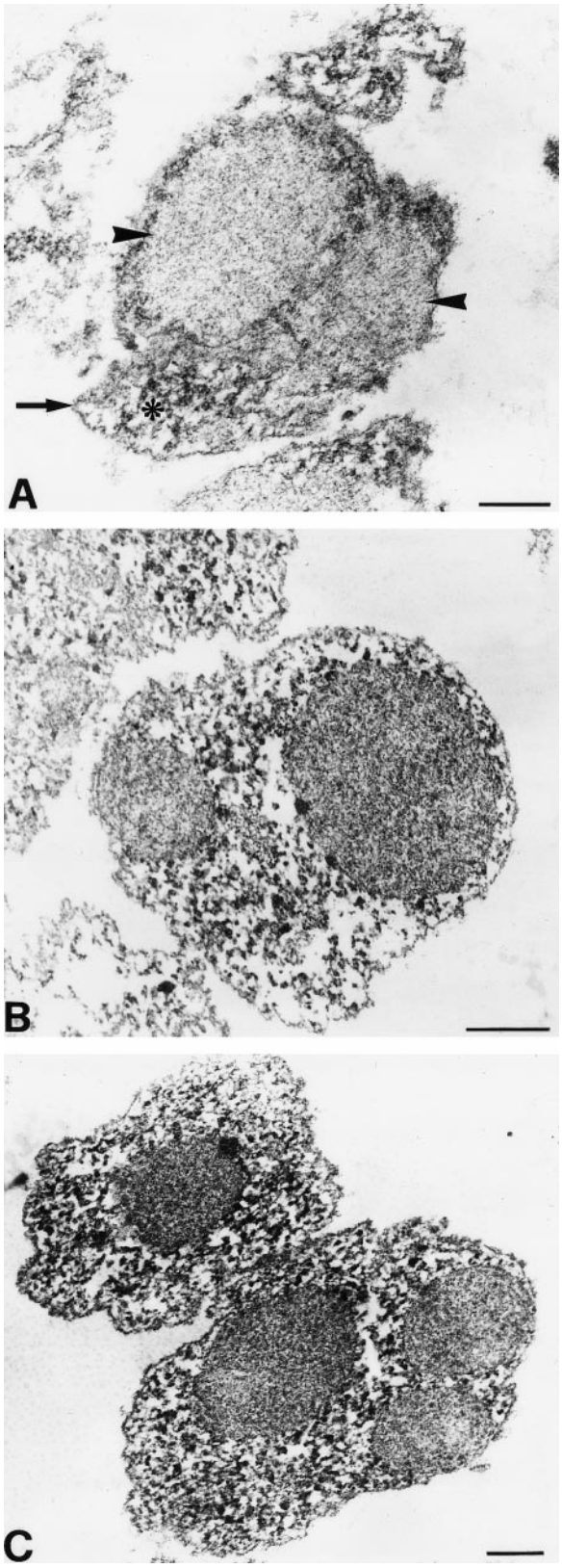



**Fig. 4.** Two-dimensional gel electrophoresis analysis of protein recovered in the nuclear matrix prepared from **(A)** control and **(B)** etoposide-treated HL-60 cells. Migration in the first dimension was from left to right. Dashed lines, pH 7.0. Molecular-weight markers are indicated on the left.




dense structures (Fig. 6B,F). We have observed similar structures in intact apoptotic cells [Zweyer et al., 1995, 1997].

### Immunofluorescent Staining

Finally, we sought to determine by immunofluorescent staining whether apoptotic nuclear matrices showed changes in the distribution of the 240-kDa protein referred to as NuMA [see He et al., 1995, for a recent review on NuMA). NuMA is cleaved early during apoptosis [Weaver et al., 1996]. Recent evidence suggests that NuMA plays a key structural role in the architecture of the normal interphase nucleus [Gueth-Hallonet et al., 1998]. To this end, we used a 240-kDa monoclonal antibody recognizing the N-terminal domain of NuMA. As shown in Figure 7, the antibody stained several spots of different size located both in the center and at the periphery of the nuclear matrices from control cells. (Fig. 7A,C,E). In apoptotic matrices, the spots were smaller if the stabilization had been carried out by either a 37°C incubation (Fig. 7B) or NaTT at 0°C (Fig. 7D). By contrast, the spots were extremely larger, bright, and less numerous if the stabilization procedure had been obtained with NaTT plus a 37°C incubation (Fig. 7F).

## DISCUSSION

Since the nuclear matrix is believed to represent the skeleton of the nucleus and to organize the functional domains of this organelle, it is very interesting to study its fate during apoptosis, i.e., a process characterized by the occurrence of dramatic changes at the nuclear level. The findings of Arends et al. [1990], who reported that the nuclear matrix is largely intact in apoptotic thymocytes, appear to contradict the proteolytic degradation of several matrix proteins, which has been subsequently reported by other investigators [see Martelli et al., 1997, for a review on this subject). However, it should be considered that an issue of fundamental importance in the field of nuclear matrix is that the different isolation procedures

**Fig. 5.** Transmission electron microscope analysis of isolated nuclear matrices from HL-60 control cells. **A:** Stabilization with 37°C, peripheral lamina (arrow), nucleolar remnants (arrowhead), fibrogranular network (*). **B:** Stabilization with NaTT at 0°C; the fibrogranular network often shows empty areas (arrow). **C:** Stabilization with NaTT plus 37°C incubation. The matrices always exhibit a well-preserved structure. Scale bar = 1 μm.

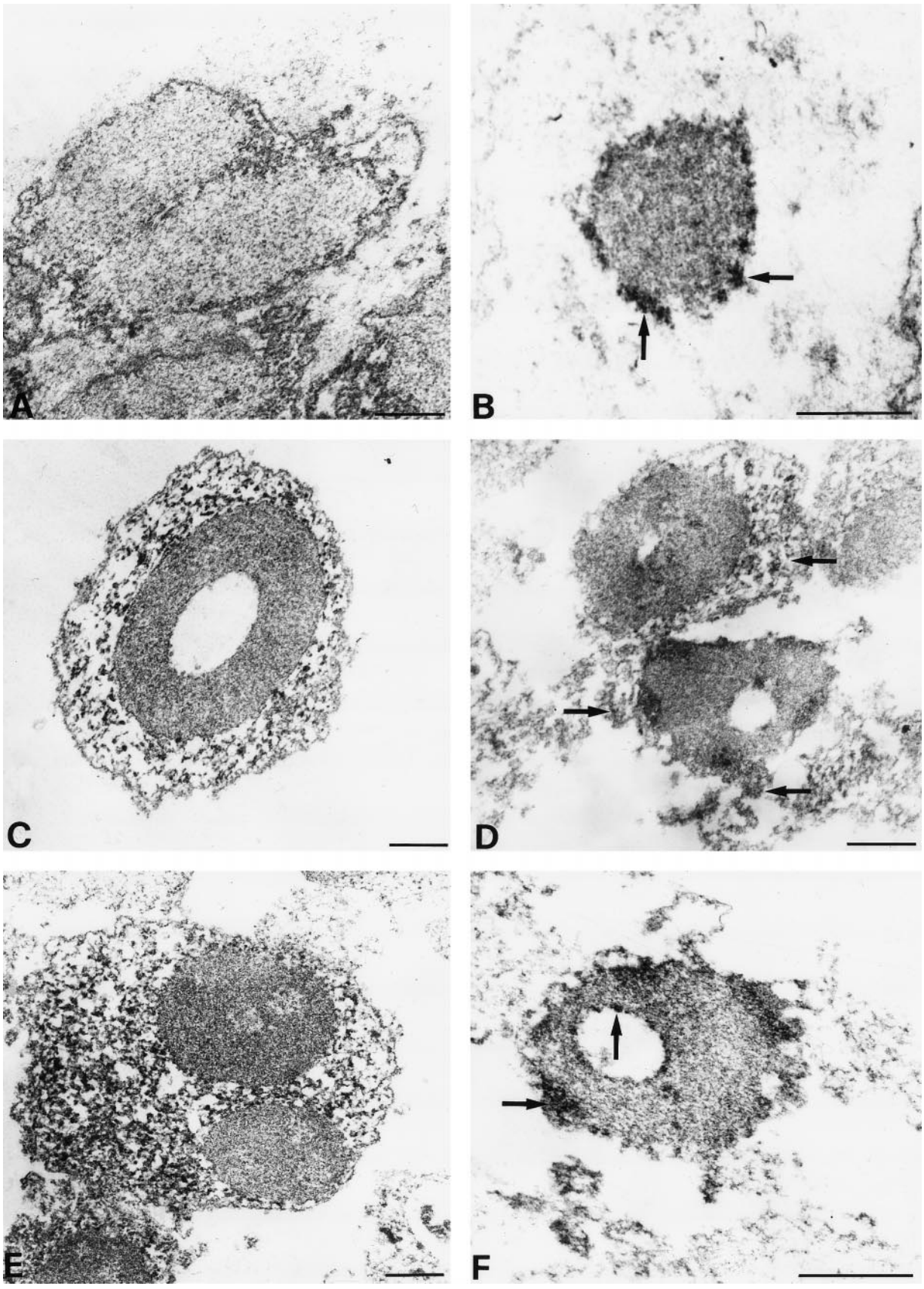



Figure 6.

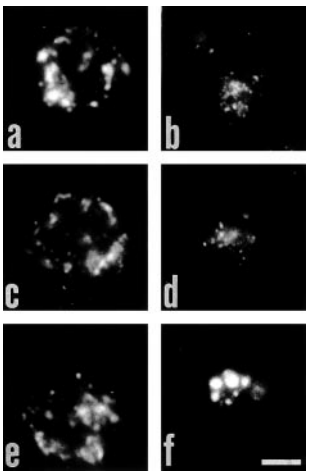



**Fig. 7.** Immunofluorescent staining for NuMA protein in isolated nuclear matrices. A,C,E: Matrices from control cells. Stabilization with **(A)** 37°C; **(C)** with NaTT at 0°C; and **(E)** with NaTT plus 37°C incubation. B,D,F: Matrices from camptothecin-treated cells. Stabilization with **(B)** 37°C; **(D)** with NaTT at 0°C; and **(F)** with NaTT plus 37°C incubation. Scale bar = 5 μm.

commonly employed to isolate the nucleoskeleton can dramatically affect both the biochemical properties and the ultrastructural morphology of the final matrices.

It should be emphasized that Arends et al. [1990] prepared the nuclear matrix from nuclei which had been exposed to NaTT and in some cases also to a 37°C incubation. Both NaTT and exposure to 37°C are known to induce a stabilization of the internal matrix network as well as of residual nucleoli [see Martelli et al., 1996]. However, there is also concern about the possible artifacts induced in the nuclear matrix structure by 37°C incubation [Martelli et al., 1996; Pederson, 1998]. We have recently reported that marked biochemical and morphological changes were detectable in the nuclear matrix prepared from apoptotic HL-60 cells [Martelli et al., 1999]. In these investigations, to isolate the matrix we employed the protocol devised by Penman and coworkers [e.g., Fey et al., 1986; He et al., 1990]. With this method, whole cells are permeabilized with a nonionic detergent to remove membranes and soluble proteins; the samples are then treated with nucleases and salt solutions to remove chromatin. At the end, the so-called nuclear matrix-intermediate filament fraction is obtained. It is worth emphasizing that with this technique we avoided the use of low-ionic strength buffers to isolate nuclei, i.e., conditions known to be a potential source for artifacts [Cook, 1988]. Moreover, there is no need to stabilize the internal network and nucleolar remnants, which are indeed preserved by the use of this technique, probably the most gentle devised so far to isolate nucleoskeletal structures [Nickerson et al., 1995]. With the above in mind, we decided to investigate to what an extent biochemical and morphological changes could be detected in the nuclear matrix prepared from apoptotic cells using more conventional techniques [Belgrader et al., 1991; Martelli et al., 1991], similar to those used by Arends et al. [1990]. Before DNase I digestion and 2 M NaCl extraction, isolated nuclei were either incubated at 0°C (a condition that does not result in stabilization of the inner network and residual nucleoli)[Martelli et al., 1991] or stabilized with chemical and/or physical agents (NaTT and moderate heat). Independent of the treatment, the nuclear matrix from apoptotic samples retained less protein than controls. SDS-PAGE analysis of polypeptide profile of nuclear matrices from apoptotic cells revealed mainly quantitative changes, as compared with those obtained from normal cells. Arends et al. [1990] claimed that they detected no differences in the polypeptide composition between normal and apoptotic matrices, but close inspection of their gels as presented in the figure of the original paper revealed to us that indeed many proteins of normal matrices were either underrepresented or absent in apoptotic matrices. Our findings were also corroborated by two-dimensional gel separations that again showed the existence of marked quantitative differences between control and apoptotic samples. Moreover, two-dimensional analysis allowed us to demon-

**Fig. 6.** Transmission electron microscope analysis of isolated nuclear matrices from camptothecin-treated HL-60 cells. Stabilization (A,B) with 37°C; (C,D) with NaTT at 0°C; and (E,F) with NaTT plus 37°C incubation. **A:** Matrices derived from cells not yet apoptotic are well conserved. **B:** A nucleolar remnant with clusters of electrondense granules at the periphery (arrows), surrounded by residues of the fibrogranular network. **C:** A nuclear matrix conceivably derived from a cell in the early stages of the apoptotic process. Note the large central cavity. Otherwise, it is very similar to the matrix depicted in Figure 5B. **D:** Two apoptotic nucleolar remnants with an electrotransparent central area and electrondense structures at the periphery. Surrounding residues of the fibrogranular network are present (arrows). **E:** Matrix isolated from a cell not yet apoptotic. **F:** Typical apoptotic nucleolar remnant surrounded by scarce residues of the fibrogranular network. Small electrondense structures are present at the periphery; they line the central cavity (arrows) as well. Scale bar = 1 μm.

strate that the changes were present with yet another chemical with a pro-apoptotic effect on HL-60 cells, i.e., etoposide. Therefore, the changes in the recovery of nuclear matrix protein seem to be a constant feature of apoptotic cell death. Nevertheless, it should be noted that the differences seen in two-dimensional gels were in some cases not as striking as in one-dimensional separations (e.g., cf. Fig. 3A with 3C). This might be because not all the nuclear matrix proteins solubilized in the lysis buffer used for two-dimensional analysis (indeed, we removed insoluble material before running the first dimension; see Materials and Methods), which might have affected the results.

Recent results by Gerner et al. [1998] also dealt with changes in the composition of nuclear matrix proteins during apoptosis of rat embryo cells. Nevertheless, in addition to the disappearance of a single spot, these authors also described the appearance of new spots at both early and late times of the apoptotic process. It was thus hypothesized that these spots might correspond to new proteins translocating from cytoplasm to the nucleus (proteases, DNases, transcription factors, protein kinases) because of their involvement in the apoptotic nuclear destruction. However, the possibility exists that these new spots correspond to fragments of degraded nuclear matrix proteins. We have indeed shown [Martelli et al., 1999] that multiple proteolytic fragments of DNA topoisomerase IIα are still present in the nuclear matrix prepared from apoptotic HL-60 cells.

Furthermore, TEM analysis was able to demonstrate changes in the morphology of nuclear matrix samples prepared from apoptotic cells. Even though they were not as striking as those we detected in apoptotic nuclear matrix samples prepared by the technique of Penman and coworkers [Martelli et al., 1999], they were nonetheless clearly recognizable. In any case, the results presented in this paper confirm that the nucleolus (or some of its constituents) is a nuclear domain very resistant to apoptotic execution [see Falcieri et al., 1994], because we detected nucleolar remnants (surrounded by residues of the inner network) still depicting a large clear inner area, similar to those we reported for intact apoptotic HL-60 cells [Zweyer et al., 1995, 1997].

Finally, also immunofluorescent staining for NuMA protein showed a different distribution of this antigen in apoptotic nuclear matrices. However, in this case it should be stressed that only when nuclei from apoptotic cells were stabilized by NaTT plus a 37°C incubation, the antibody to NuMA stained very large and bright dots, now considered a typical maker for intact apoptotic cells [Sodja et al., 1997, 1998]. When nuclei were stabilized with either NaTT alone or 37°C incubation alone, the dots were much smaller. This probably indicates that these two stabilizing treatments are insufficient to preserve the original distribution of NuMA protein present in intact apoptotic cells. The use of the Penman's technique preserved the typical distribution of NuMA in apoptotic matrices [Martelli et al., 1999].

In conclusion, our data strengthen the contention that depolymerization of the nuclear matrix occurs during apoptotic cell death. The biochemical changes in nuclear matrices prepared by apoptotic cells are largely independent of the preparation techniques employed to isolate the nuclear matrix, and they were also seen if a different inducer (etoposide) was employed. As far as the morphological changes are concerned, they were also detectable with all types of stabilization, whereas the preservation of the distribution of a single nuclear matrix protein (NuMA) seems to be more sensitive to the use of a particular isolation protocol.

In any case, for an optimal preservation of the very delicate apoptotic matrix structures, the use of a very gentle technique, such as that pionereed by Penman and coworkers, would seem desirable.

## ACKNOWLEDGMENTS

This work was supported by Fondi A.I.R.C. (Associazione Italiana per la Ricerca sul Cancro) 1997 (to A.M.M.), and by 60% grant from Università di Trieste. We thank Dr. R.L. Ochs for the kind gift of the antibody and Giovanna Baldini for the illustrations.

## REFERENCES

Arends MJ, Morris RG, Wyllie AH. 1990. Apoptosis. The role of endonuclease. Am J Pathol 136:593–608.

Belgrader P, Siegel AJ, Berezney R. 1991. A comprehensive study on the isolation and characterization of the HeLa S3 nuclear matrix. J Cell Sci 98:281–291.

Bellamy OC, Malcomson DG, Harrison DJ, Wyllie AH. 1995. Cell death in health and disease: the biology and regulation of apoptosis. Semin Cancer Biol 6:3–16.

Berezney R, Mortillaro M J, Ma H, Wei X, Samarabandu J. 1995. The nuclear matrix: a structural milieu for genomic function. Int Rev Cytol 162A:1–65.

Bode J, Stengert-Iber M, Kay V, Schalke T, Dietz-Pfeilstetter A. 1996. Scaffold/matrix-attached regions: topological switches with multiple regulatory functions. Crit Rev Eukar Gene Expr 6:115–138.

Boulikas T. 1995. Chromatin domains and prediction of MAR sequences. Int Rev Cytol 162A:279–388.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254.

Casiano CA, Martin SJ, Green DR, Tan EM. 1996. Selective cleavage of nuclear autoantigens during CD95(Fas/APO-1)-mediated T cell apoptosis. J Exp Med 184:765–770.

Cohen GM. 1997. Caspases: the executioners of apoptosis. Biochem J 326:1–16.

Collins JA, Schandl CA, Young KK, Vesely J, Willingham MC. 1997. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem 45:923–934.

Cook PR. 1988. The nucleoskeleton: artefact, passive framework or active site? J Cell Sci 90:1–6.

Del Bino G, Lassota P, Darzynkiewicz Z . 1991. The S-phase cytoxicity of camptothecin. Exp Cell Res 193:27–35.

Earnshaw WC. 1995. Nuclear changes in apoptosis. Curr Opin Cell Biol 7:337–343.

Falcieri E, Zamai L, Santi S, Cinti C, Gobbi P, Bosco D, Cataldi A, Betts C, Vitale M. 1994. The behaviour of nuclear domains in the course of apoptosis. Histochemistry 104:221–231.

Fey EG (1986) Krochmalnic G, Penman S. 1986. The non-chromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. J Cell Biol 102:1654–1665.

Fields AP, Kaufmann S H, Shaper J H. 1986. Analysis of the internal matrix. Oligomers of a 38 kD nucleolar polypeptide stabilized by disulfide bonds. Exp Cell Res 164:139–153.

Gerner C, Seelos C, Sauermann G. 1998. Alteration of nuclear matrix protein composition during apoptosis in rat embryo cells. Exp Cell Res 238:472–480.

Gueth-Hallonet C, Wang J, Weber K, Osborn M. 1998. Induction of a regular nuclear lattice by overexpression of NuMA. Exp Cell Res 243:434–452.

Hale A J, Smith CA, Sutherland LC, Stoneman VEA, Longthorne VL, Culhane AC, Williams GT. 1996. Apoptosis: molecular regulation of cell death. Eur J Biochem 236: 1–26.

He D , Nickerson JA, Penman S. 1990. Core filaments of the nuclear matrix. J Cell Biol 110:569–580.

He D, Zeng C, Brinkley BR. 1995. Nuclear matrix proteins as structural and functional components of the mitotic apparatus. Int Rev Cytol 162B:1–74.

Jacobson MD, Weil M, Raff MC. 1997. Programmed cell death in animal development. Cell 88:347–354.

Kroemer G, Petit P, Zamzami N, Vayssière J-L, Mignotte B. 1995. The biochemistry of programmed cell death. FASEB J 9:1277–1287.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the bacteriophage T4. Nature 227:680–685.

Lagarkova MA, Iarovaia OV, Razin SV. 1995. Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers. J Biol Chem 270:20239–20241.

Leist M, Nicotera P. 1997. The shape of cell death. Biochem Biophys Res Commun 236:1–9.

Martelli AM, Falcieri E, Gobbi P, Manzoli L, Gilmour RS, Cocco L. 1991. Heat-induced stabilization of the nuclear matrix. A biochemical and morphological analysis in mouse erythroleukemia cells. Exp Cell Res 196:216–225.

Martelli AM, Manzoli L, Rubbini S, Billi AM, Bareggi R , Cocco L. 1995. The protein composition of Friend cell nuclear matrix stabilized by various treatment. Different recovery of nucleolar proteins B23 and C23 and nuclear lamins. Biol Cell 83:15–22.

Martelli AM, Cocco L, Riederer BM, Neri LM. 1996. The nuclear matrix: a critical appraisal. Histol Histopathol 11:1035–1048.

Martelli AM, Bareggi R, Bortul R, Grill V, Narducci P, Zweyer M. 1997. The nuclear matrix and apoptosis. Histochem Cell Biol 108:1–10.

Martelli AM, Bortul R, Fackelmayer FO, Tazzari PL, Bareggi R, Narducci P, Zweyer M. 1999. Biochemical and morphological characterization of the nuclear matrix from apoptotic HL-60 cells. J Cell Biochem 72:35–46.

Martins LM, Kottke T, Mesner PW, Basi GS, Sinha S, Frigon N, Tatar E, Tung JS, Bryant K, Takahashi A, Svingen PA, Madden BJ, McCormick DJ, Earnshaw WC, Kaufmann SH. 1997. Activation of multiple interleukin-1β converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. J Biol Chem 272:7421–7430.

Miller TE, Beausang LA, Meneghini M, Lidgard G. 1993. Death-induced changes to the nuclear matrix: the use of anti-nuclear matrix antibodies to study agents of apoptosis. Biotechniques 15:1042–1047.

Neri LM, Santi S, Marugg RA , Riederer BM, Capitani S, Cataldi A, Martelli AM. 1994. In vitro heat exposure induces a redistribution of nuclear matrix proteins in K562 erythroleukemia cells. Exp Cell Res 213:275–285.

Neri LM, Fackelmayer FO, Zweyer M, Kohwi-Shigematsu T, Martelli AM. 1997. Subnuclear localization of S/MAR-binding proteins is differently affected by in vitro stabilization with heat or $Cu^{++}$. Chromosoma 106:81–93.

Nickerson JA, Blencowe BJ, Penman S. 1995. The structural organization of nuclear metabolism. Int Rev Cytol 162A:67–123.

O'Farrell PH. 1975. High-resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021.

O'Farrell PZ, Goodman HM, O'Farrell PH. 1977. High-resolution two-dimensional electrophoresis of basic as well acidic proteins. Cell 12:1133–1142.

Pederson T. 1998. Thinking about a nuclear matrix. J Mol Biol 277:147–159.

Sodja C, Walker PR, Brown DL, Chaly N. 1997. Unique behaviour of NuMA during heat-induced apoptosis of lymphocytes. Biochem Cell Biol 75:399–414.

Sodja C, Walker PR, Brown DL, Chaly N. 1998. Splenic T lymphocytes die preferentially during heat-induced apoptosis: NuMA reorganization as a marker. J Cell Sci 111: 2305–2313.

Thornberry NA, Rosen A, Nicholson DW. 1997. Control of apoptosis by proteases. Adv Pharmacol 41:155–177.

Tinnemans MMFJ, Lenders M-HJH, ten Velde GPM, Ramaekers FCS, Schutte B. 1995. Alterations in cytoskeletal and nuclear matrix-associated proteins during apoptosis. Eur J Cell Biol 68:35–46.

Vaux DL, Strasser A. 1996. The molecular biology of apoptosis. Proc Natl Acad Sci USA 93:2239–2244.

Villa P, Kaufmann SH, Earnshaw WC. 1997. Caspases and caspase inhibitors. Trends Biochem Sci 22:388–393.

Weaver VM, Carson CE, Walker PR, Chaly N, Lach B, Raymond Y, Brown DL, Sikorska M. 1996. Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. J Cell Sci 109:45–56.

Zweyer M, Bareggi R, Grill V, Soranzo MR, Marugg RA, Riederer BM, Narducci P, Martelli AM. 1995. Behaviour of nuclear matrix proteins during camptothecin-induced apoptosis in HL-60 human leukemia cells. Exp Cell Res 221:27–40.

Zweyer M, Riederer BM, Ochs RL, Fackelmayer FO, Kohwi-Shigematsu T, Bareggi R, Narducci P, Martelli AM. 1997. Association of nuclear matrix proteins with fibrogranular nuclear bodies in cell lines undergoing apoptosis. Exp Cell Res 230:325–336.