

# Release of Hypoacetylated and Trimethylated Histone H4 Is an Epigenetic Marker of Early Apoptosis\*<sup>§</sup>

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**Nuclear events such as chromatin condensation, DNA cleavage at internucleosomal sites, and histone release from chromatin are recognized as hallmarks of apoptosis. However, there is no complete understanding of the molecular events underlying these changes. It is likely that epigenetic changes such as DNA methylation and histone modifications that are involved in chromatin dynamics and structure are also involved in the nuclear events described. In this report we have shown that apoptosis is associated with global DNA hypomethylation and histone deacetylation events in leukemia cells. Most importantly, we have observed a particular epigenetic signature for early apoptosis defined by a release of hypoacetylated and trimethylated histone H4 and internucleosomal fragmented DNA that is hypermethylated and originates from perinuclear heterochromatin. These findings provide one of the first links between apoptotic nuclear events and epigenetic markers.**

Apoptosis is a form of cell death essential for the morphogenesis, development, differentiation, and homeostasis of eukaryotic multicellular organisms. The activation of a genetically controlled cell death program leading to apoptosis results in characteristic biochemical and morphological features that take place both outside and inside the nucleus (1). The biochemical mechanisms responsible for key nuclear events, such as chromatin condensation, DNA fragmentation, and release of nuclear proteins, although commonly used as markers for apoptosis, are not fully understood (2). The regulated nature of apoptosis makes it likely that nuclear changes experienced by apoptotic cells are mediated by epigenetic markers. This epigenetic information is basically stored as DNA methylation and post-translational histone modifications. These two groups of modifications play an active role in organizing, compartmentalizing, and regulating genetic information encoded in DNA by defining nuclear architecture, and gene expression (3). With regard to gene regulation, the major functional consequence of DNA methylation is the repression of transcription (4). In the case of

histone modifications, the type of modification (acetylation, methylation, phosphorylation, etc.) and the specific amino acid residue that is modified determine the functional effect. Histone modifications also determine the nature of chromatin regions, such as heterochromatin. For example, the inactive X chromosome is characterized by trimethylation of Lys-27 of H3 and dimethylation of Lys-9 of H3 (5–7), whereas Lys-9 trimethylation and Lys-27 monomethylation of H3 and Lys-20 trimethylation of H4 are characteristic of pericentric heterochromatin (8, 9).

In the context of apoptosis, DNA fragmentation and chromatin condensation have been associated with changes in histone modifications (10, 11). For instance, apoptotic phosphorylation of histone H2A, H2B, and H3, dephosphorylation of histone H1, and H2A deubiquitylation (12–16) have been associated with DNA fragmentation and chromatin condensation. These results suggest that a conformational change in chromatin structure is needed prior to condensation, although the mechanism and functional significance remain unclear. A global decrease in histone acetylation in apoptosis has also been reported (17, 18), although some authors have interpreted this result to be a loss of hyperacetylated histones by degradation rather than the consequence of the active hypoacetylation of histones during apoptosis (19). Thus, there is a need to clarify the role of these modifications in different nuclear events during apoptosis.

Regarding apoptotic release of histones and other nuclear proteins, it has been proposed that this process is associated with chromatin condensation and DNA fragmentation (20, 21). Histone release may not merely be a simple by-product of chromatin condensation or DNA fragmentation and could be the result of specific chromatin modifications in particular nuclear compartments. In fact, during apoptosis a series of nuclear matrices and membrane proteins that are fundamental to the maintenance of internal nuclear structures are degraded by caspases (22–24). For instance, degradation of nuclear lamins, which maintain nuclear structure by attaching chromatin to the nuclear membrane through heterochromatic structures (25, 26), coincides with chromatin condensation and DNA fragmentation in apoptotic cells. Although the breakdown of internal nuclear structures is a prerequisite for chromatin condensation and DNA fragmentation (27, 28), it has been proposed that the maintenance of high order chromatin structures is essential for proper chromatin condensation (29). In fact, drugs that modify chromatin structure are able to block apoptotic chromatin condensation (30).

In this report, we have focused our attention on the global characterization of histone modification and DNA methylation changes associated with the apoptotic process, in particular in connection with the release of nuclear material to the cytosol. Significant global deacetyla-

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tion of histones H3 and H4 associated with its release during apoptosis has been observed. Most interestingly, we have identified a unique pattern of post-translational modifications that is characteristic of histones released early on in apoptosis. Released histone H4 is specifically hypoacetylated and trimethylated at Lys-20, whereas released histone H3 is hypoacetylated, demethylated, and dephosphorylated. These histones are released and cofractionate with internucleosomally fragmented DNA with a greater 5-methylcytosine content than the DNA that remains in the nucleus. This released DNA originates in the heterochromatic perinuclear region of the cell. These data, together with the observed pattern of modification of released histones, suggest that the released material results from the degradation of perinuclear heterochromatin and internal structures of the nucleus early on in apoptosis.

## EXPERIMENTAL PROCEDURES

**Materials**—Jurkat and HL60 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. The following rabbit polyclonal antibodies were used: anti-histone H3 (Abcam) directed against a C-terminal peptide of H3, anti-dimethyl Lys-4 histone H3 (Upstate Biotechnologies, Inc.), anti-dimethyl Lys-9 histone H3 (Upstate Biotechnologies, Inc.), anti-phospho Ser-10 histone H3 (Upstate Biotechnologies, Inc.), anti-acetyl histone H3 (Upstate Biotechnologies, Inc.), anti-acetyl Lys-9 histone H3 (Abcam), anti-acetyl histone H4 (Upstate Biotechnologies, Inc.), anti-acetyl Lys-5 histone H4 (Abcam), anti-acetyl Lys-8 histone H4 (Abcam), anti-acetyl Lys-12 histone H4 (Upstate Biotechnologies, Inc.), and anti-acetyl Lys-16 histone H4 (Upstate Biotechnologies, Inc.). Camptothecin and etoposide (Sigma) were used as apoptosis inducers. Apoptosis was analyzed using the Vybrant® apoptosis assay kit, 4-YO-PRO®-1/propidium iodide (Molecular Probes/Invitrogen). This kit is based on the use of the green fluorescent YO-PRO®-1 dye that specifically stains apoptotic cells that remain impermeant to propidium iodide (a dead cell stain). Live cells are not stained with YO-PRO®-1. Cells are then sorted by flow cytometry.

**Induction of Apoptosis**—Jurkat and HL60 cells were exposed to 100  $\mu$ M etoposide or 2  $\mu$ g/ml of camptothecin and incubated for 3–8 h (30). Apoptosis was monitored by flow cytometry.

**Isolation of Histones**—Histones were extracted from cell pellets by acid extraction on 0.25 M HCl followed by acetone precipitation (31). To investigate the release of histone to the cytosolic fraction during apoptosis, we performed hypotonic lysis with 20 mM pH 8.0 buffer containing 150 mM NaCl and 1% Triton X-100 as described in Wu *et al.* (20). Two fractions are obtained in this case: a cell lysate, containing the cytosolic fraction, and a nuclear pellet. In this case, the supernatant corresponding to the cytosolic fraction was precipitated using 20% trichloroacetic acid on ice for 30 min, centrifuged at 4 °C for 10 min, and washed once with acetone. Histones were then obtained by acid extraction as described above.

**Quantification of Global Histone Acetylation by High-performance Capillary Electrophoresis**—The degree of histone acetylation was quantified by a modification of a previously described method (32, 33). Individual histones were fractionated by reversed-phase high-performance liquid chromatography (HPLC)<sup>4</sup> on a Delta-Pak C18 column (Waters) eluted with an acetonitrile gradient (20–60%) in 0.3% trifluoroacetic acid (34) using a Beckman HPLC gradient system. Purity of histones was measured by PAGE. The non-, mono-, di-, tri-, and tetraacetylated his-

tone derivatives of H3 fraction were resolved by high-performance capillary electrophoresis (HPCE). A non-coated fused silica capillary (Beckman-Coulter) (60.2 cm  $\times$  75.0  $\mu$ m, effective length 50.0 cm) was used in a CE system (P/ACETM MDQ; Beckman-Coulter) connected to a data processing station (32 Karat™ Software). The running buffer was 110 mM phosphate buffer, pH 2.0, containing 0.03% (w/v) hydroxypropylmethyl-cellulose. Running conditions consisted of a temperature of 25 °C and an operating voltage of 12 kV. On-column absorbance was monitored at 214 nm. Before each run, the capillary system was conditioned by washing with 0.1 M NaOH for 3 min and 0.5 M H<sub>2</sub>SO<sub>4</sub> for 2 min and equilibrated with the running buffer for 3 min. Samples were injected under pressure (0.3 psi) for 3 s. Samples were obtained in triplicate, and all samples were analyzed in duplicate. Error bars in graphs represent standard deviation.

**Western Blotting**—Histones were separated on 15% SDS-PAGE gel and blotted onto a polyvinylidene difluoride membrane of 22- $\mu$ m pore size (Immobilon PSQ; Millipore). The membrane was blocked in 5% milk PBS-T (phosphate-buffered saline with 0.1% Tween-20) and immunoprobed with antibodies raised against different peptides containing different histone modifications as described above. The secondary antibodies used were goat anti-rabbit conjugated to horseradish peroxidase (1:3000) (Amersham Biosciences) and sheep anti-mouse horseradish peroxidase (1:3000). Bands obtained in Western blot were scanned and analyzed by Quantity One software (Gel Doc 2000; Bio-Rad). Experiments were performed in triplicate. Semiquantitative significance of the differences was estimated by direct comparison of the obtained values.

**Mass Spectrometry Analysis of Histones**—Histone H4 global acetylation and acetylation at the specific lysine 16 site were analyzed by mass spectrometry. We separated acid-extracted histones by SDS-PAGE, excised the Coomassie-stained bands corresponding to histone H4, subjected them to acetylation with D6-acetic anhydride, and finally digested with trypsin as previously described (35). Supernatants were collected, vacuum-dried, and redissolved in 0.5 ml of 0.1% trifluoroacetic acid.

Matrix-assisted laser desorption ionization time-of-flight MS analysis of the samples was carried out in a mass spectrometer Autoflex (Bruker Daltonics) in a positive ion reflector mode. Samples were added to a matrix consisting of 0.5 ml of 5 mg/ml of 2,5-dihydroxybenzoic acid in water:acetonitrile (2:1) with 0.1% trifluoroacetic acid. The ion acceleration voltage was 20 kV. Each spectrum was internally calibrated with the masses of two trypsin autolysis products.

MS/MS analyses were performed in a linear LTQ ion trap mass spectrometer (Thermo Finnigan) equipped with a nano-electrospray ionization source by using coated GlassTip PicoTip emitters (New Objective). Samples were desalted and concentrated with Zip Tips (Millipore, Bedford, MA) following the manufacturer's protocol. The spectrometer was operated according to the manufacturer's instructions with manual adjustment of the collision energies. Fragment spectra were interpreted manually.

**Global 5-Methylcytosine Quantification**—The 5-methylcytosine (mC) content was quantified by HPCE as previously described (36). In brief, DNA samples were speed-back preconcentrated to 0.1 mg/ml and enzymatically hydrolyzed in a final volume of 5 ml. Samples were then directly injected into a Beckman MDQ high-performance capillary electrophoresis apparatus, and mC content was determined as the percentage of mC of total cytosine: mC peak area  $\times$  100/(C peak area + mC peak area). Error bars in graphs represent standard deviation. Data are representative of three independent experiments.

<sup>4</sup> The abbreviations used are: HPLC, high performance liquid chromatography; HPCE, high-performance capillary electrophoresis; MS, mass spectrometry; mC, methylcytosine.

## An Epigenetic Marker of Early Apoptosis

**Competitive Hybridization of Apoptotic DNA Fractions in Metaphase Chromosomes**—To study the distribution along the chromosomes of the DNA isolated from nuclear pellet and cell lysate fractions, we modified the competitive genomic hybridization strategy (37). In this hybridization, we compared the DNA isolated from each of the fractions (either the cell lysate or the nuclear pellet) with the DNA isolated from intact cells. The DNA isolated from each of the fractions was labeled with Spectrum Red dUTP by CGH nick-translation kit (Vysis, Inc., Downer Grov, IL), and the total genomic DNA was labeled with Spectrum Green. The metaphases were captured using a fluorescence microscope (Olympus BX60) equipped with a CCD camera (Photometrics Sensys camera) and then analyzed using the chromofluor image analysis system (Cytovision; Applied Imaging Ltd, Newcastle, UK). 13–25 chromosomes were analyzed for each hybridization.

**Staining with Fluorescent DNA Probes and Fluorescence Microscopy**—Control cells were fixed in 3.7% formaldehyde for 30 min at room temperature or methanol (−20 °C) for 1 min and permeabilized with phosphate-buffered saline-0.5% Triton X-100 for 10 min at room temperature as previously described (38). DNA from the cell lysate fraction labeled with Spectrum Red was used to hybridize fixed cells. Confocal optical sections were obtained using a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg GmbH) equipped with krypton and argon lasers, and images were processed using Adobe Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA).

## RESULTS

**Global Reduction in Histone Acetylation Occurs during Apoptosis**—As a first screening approach to investigating a potential connection between epigenetic alterations and nuclear changes in apoptosis, we analyzed global changes in the modification status of histones during apoptosis by Western blot. A change in the pattern of histone modifications could be associated with morphological changes that occur in the nucleus in apoptosis. We treated Jurkat cells with etoposide and acid extracted their histones at different times. For each sample, the level of apoptosis was monitored by flow cytometry (Fig. 1A, *bottom*). Antibodies against the global hyperacetylated forms of histones H3 and H4 and others against the monoacetylated form at specific residues of histone H3 (Lys-9) and histone H4 (Lys-5, -8, -12, and -16) were used. Antibodies against other modifications, such as phospho-Ser-10, dimethyl-Lys-4 and dimethyl-Lys-9 of histone H3, were also included.

We first observed a significant decrease in global levels of histone H4 acetylation in apoptotic samples (Fig. 1A). However, when looking at specific lysine residues of H4 only Lys-8 and Lys-16 exhibited a loss of acetylation during apoptosis. No significant variations of acetylation were observed at Lys-5 and Lys-12. These results are consistent with previous reports that also suggest that the acetylation patterns of the pairs Lys-8 and Lys-16, on one hand, and Lys-5 and Lys-12, on the other, are coupled (39).

In contrast to H4, no significant changes in acetylation (either global or site-specific) were observed for histone H3. Also, phosphorylation of Ser-10 and methylation of Lys-4 and Lys-9 of H3 appeared to remain stable (Fig. 1A).

Because the only changes observed were associated with acetylation status, an alternative quantitative approach was used to estimate changes in histone acetylation of H3 and H4. The method requires the fractionation of histones by reversed phase HPLC followed by HPCE separation (35–37). This allows the resolution and quantification of all acetylated forms of histones H3 and H4. In this analysis, for histone H4 each of the non-, mono-, di-, tri-, and tetraacetylated histone derivatives appeared as a doublet of peaks (Fig. 1B, *top panel*). The first and second

peaks of the doublet had been previously identified as di- and trimethyl-Lys-20 histone H4, respectively (40). Trimethylation of Lys-20 in H4 is a marker of constitutive heterochromatin (9, 41, 42) and aging (43).

In Jurkat cells, relative losses of about 8 and 4% in the acetylated species of H4 were observed concomitantly with equivalent increases in non-acetylated forms for the etoposide (70% apoptosis after 8 h) and camptothecin (56% apoptosis after 8 h) treatments, respectively. The results obtained for HL60 cells also showed relative losses of 10 and 5% of acetylated H4 for the etoposide (78% apoptosis after 8 h) and camptothecin (60% after 8 h) treatments, respectively (Fig. 1C, *bottom panel*).

In the case of histone H3, treatment of HL60 cells with etoposide and camptothecin resulted in respective relative losses of 5 and 3% of acetylated forms (Fig. 1C, *bottom panel*). These decreases were concomitant with an increase in the non-acetylated form of H3. Identical results were obtained with Jurkat cells (data not shown). These small decreases had not been observed when using Western blot to detect changes in the acetylation status of H3, although the HPCE analysis indicated that this is a very reproducible result. On the other hand, it is possible that the acetyl-H3 antibody does not detect all acetylated forms (mono-, di-, tri- and tetra-) that are quantified by HPCE. At any rate, the decrease in acetylation of H3 was significantly smaller than the variations observed for H4.

**Histone H4 Released from Nuclei Early in Apoptosis Is Hypoacetylated and Trimethylated**—Having analyzed the global changes in histone H3 and H4 modifications, we next compared the specific modifications exhibited by histones that are massively released from the nucleus during apoptosis (23) with those that are retained. Thus, we induced apoptosis with etoposide and camptothecin and treated the cells with a lysis buffer containing 1% Triton X-100 (see “Experimental Procedures”). Under these conditions, in which cells are lysed but the integrity of nucleus is preserved (20, 44), it is possible to separate histones released to the cytosol in apoptosis from those remaining in the chromatin. Therefore, each sample yielded two fractions that we call cell lysate, corresponding to the cytosolic fraction and nuclear pellet (Fig. 2).

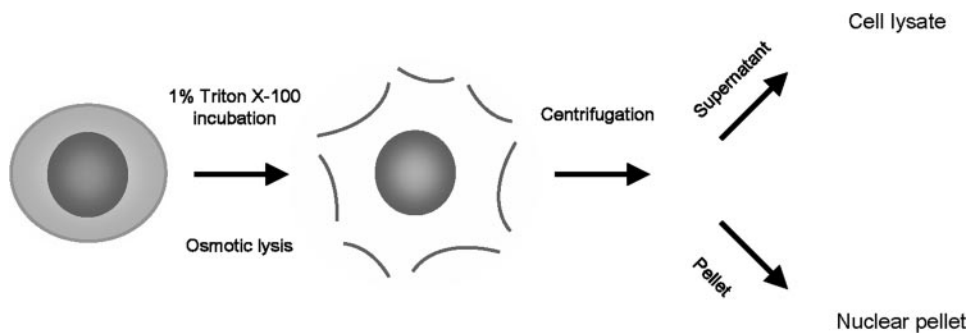
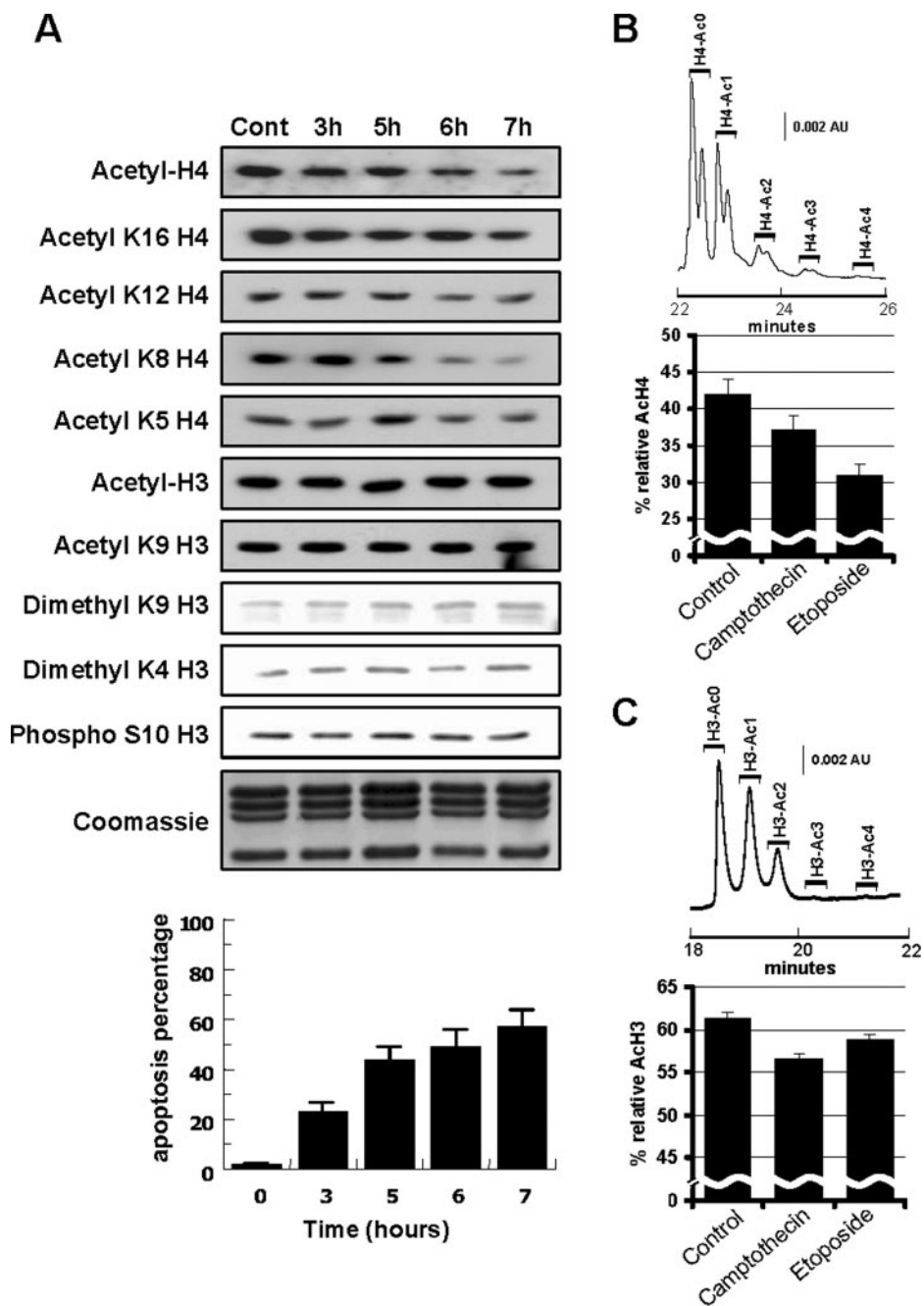
Histones were virtually absent from the cell lysate of uninduced control samples (Fig. 3A). In apoptotic samples, we observed a time-dependent increase of histones in the cell lysates, which were in fact released from the nucleus during apoptosis. The amount of histones reached about a quarter of that remaining in the pelleted fraction 8 h after inducing apoptosis (Fig. 3A).

The analysis of H4 by HPCE showed that histones retained in the nuclear pellet did not exhibit significant differences between control and apoptotic samples (Fig. 3B), unlike the results obtained when total histone H4 had been analyzed where there were 5–10% decreases in acetylated histone H4 (Fig. 1B).

However, a comparison of cell lysates with their corresponding nuclear pellets showed striking differences in the modification pattern of histone H4. Histone H4 isolated from cell lysates was found to be consistently hypoacetylated compared with its counterpart in apoptotic nuclear pellets, with an up to 15% relative decrease in the acetylated form of histone H4 during early apoptosis (Fig. 3B). Interestingly, differences in H4 acetylation between released and retained histone H4 became smaller at longer incubation times. Thus, shortly after apoptosis was induced, released histone H4 was enriched in hypoacetylated forms and the proportion of acetylated forms increased as apoptosis advanced, and the released histones more closely resembled those retained in the nuclear pellet.

However, the most striking finding about histone H4 modification profiles was that released and retained H4 fractions exhibited different patterns of methylation. Most specifically, the characteristic doublets

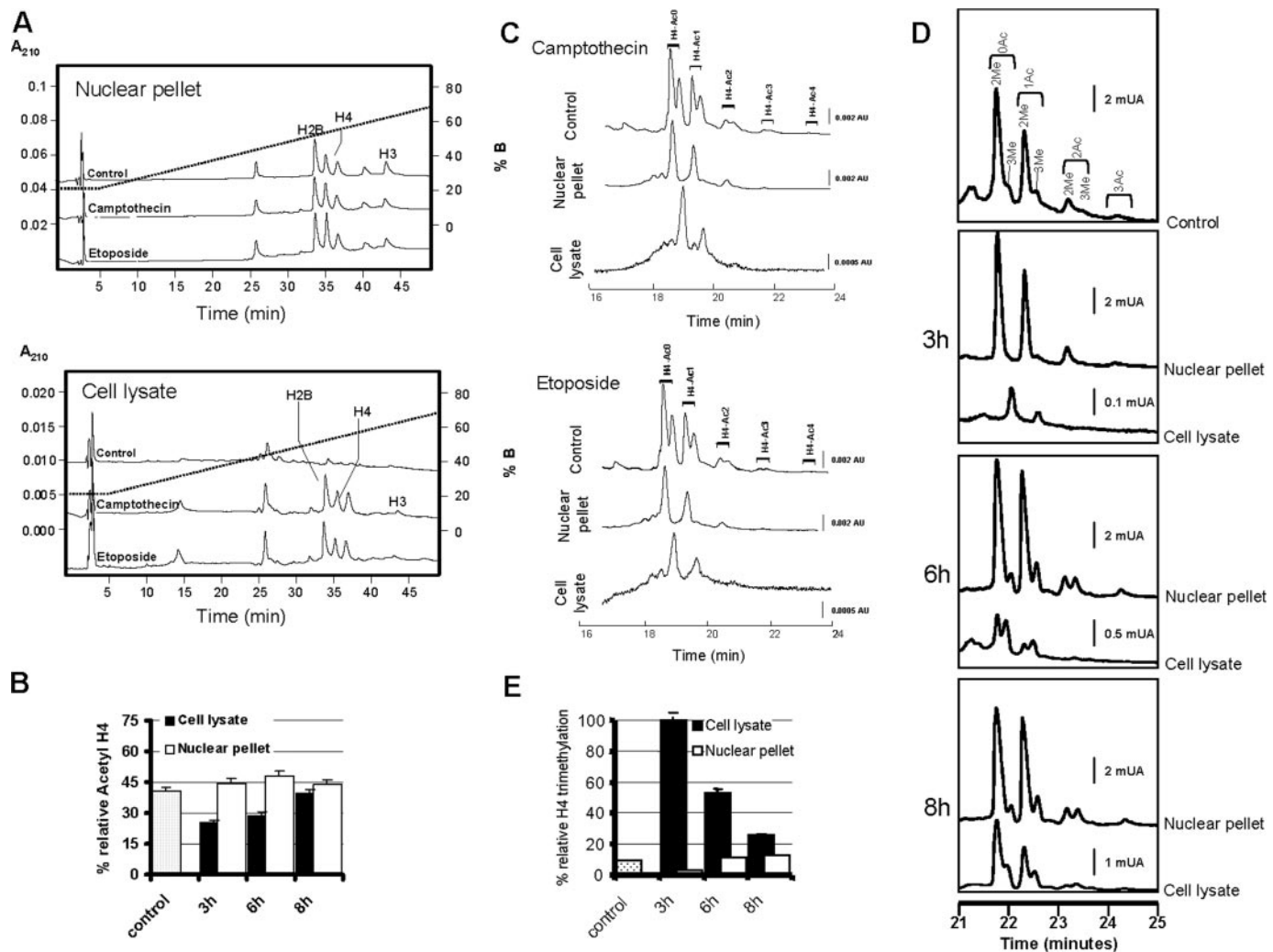
**FIGURE 1. Global analysis of histone modifications during apoptosis.** A, analysis by Western blot with antibodies against acetyl-H4, acetyl Lys-16, -12, -8, and -5 H4, and acetyl-H3, acetyl Lys-9 H3, dimethyl Lys-9 H3, dimethyl Lys-4 H3, and phospho Ser-10 H3 of histones extracted from HL60 cells at different times after apoptosis induction with etoposide. Coomassie staining is included as a loading control. The bottom panel shows the apoptosis levels quantified by flow cytometry using YO-PRO (see "Methods"). Error bars in graphs represent S.D. Data are representative of three independent measurements. B, the top panel shows an example of HPCE separation of acetylated species of histone H4 from HL60 cells. Each acetylated form appears as a doublet corresponding to two different degrees of methylation as explained under "Results." The bottom panel shows a quantification of the acetylation of histone H4 in HL60 cells treated for 8 h with camptothecin and etoposide. C, the top panel shows an example of HPCE separation of acetylated species of histone H3 from HL60 cells. The bottom panel shows a quantification of the acetylation of histone H3 in HL60 cells treated for 8 h with camptothecin and etoposide.



**FIGURE 2. Scheme depicting the protocol designed to isolate histones that are released during apoptosis.** A control cell is shown. Cells are treated with a low salt buffer containing 1% Triton X-100. Under these conditions, in which cells are lysed but the integrity of nucleus is preserved (20), two fractions are obtained by centrifugation: cell lysate, corresponding to the cytosolic fraction, and nuclear pellet. In apoptotic cells, a fraction of histones, released from chromatin, is present in the cell lysate, and nuclear pellets contain nuclei together with apoptotic bodies.

corresponding to different methylation forms of histone H4 were absent from both fractions, and whereas the cell lysate exhibited only the trimethylated form of histone H4, the nuclear pellet only contained the

dimethylated form for short times after apoptosis induction (Fig. 3C). This is evident from the comparison of the electropherograms of these two samples, where each of the single peaks that corresponds to the di-



**FIGURE 3. Analysis of histones that are released from the nucleus in apoptosis.** *A*, histone fractionation by reversed phase HPLC of nuclear pellet (top panel) and apoptotic cell lysate (released histone fraction, bottom panel) samples from HL60 control treated with etoposide and camptothecin. *B*, analysis of histone H4 acetylation of cell lysates and nuclear pellet samples after 3, 6, and 8 h of etoposide treatment. *C*, HPCE profiles of histone H4 from nuclear pellets and apoptotic cell lysates 3 h after apoptosis induction. *D*, time course analysis of the HPCE profiles of histone H4 from cell lysates and nuclear pellets (3, 6, and 8 h after etoposide treatment). H4 HPCE profiles from control samples are also shown in the top panel. *E*, relative levels of histone H4 trimethylation from nuclear pellets and cell lysates at 3, 6, and 8 h after etoposide treatment of HL60 cells.

and trimethylated forms of H4 had been fractionated during the isolation of the released histones (Fig. 3C).

These data indicated that 3 h after apoptosis induction, almost 100% of histone H4 was trimethylated in the cell lysate (Fig. 3, D and E). This specific release of trimethylated H4 was progressively lost as apoptosis advanced. Indeed, after 6 and 8 h of incubation the relative trimethylation of released histone H4 dropped to 50 and 25%, respectively, due to the release of dimethylated H4 species (Fig. 3E).

Therefore, trimethylation and hypoacetylation of histone H4 are specific features of the histone fraction released from nuclei during early apoptosis. This specific enrichment of trimethylated and hypoacetylated histones in the released fraction is progressively lost at later stages after induction of apoptosis, when released histones increasingly more resemble the histones remaining in the nuclei.

*Acetylation Status of Different Residues of Histone H3 and H4 Released during Apoptosis by Western Blot Analysis and Mass Spectrometry*—To better characterize the acetylation status of released histones, we performed Western blot analysis using antibodies against global hyperacetylated histones H3 and H4 and compared apoptotic cell lysates with nuclear pellets. We also used specific antibodies against

different lysine residues in order to investigate changes in acetylation at particular sites.

First, a significant decrease of global acetylation, both for histone H3 and H4, was observed in cell lysates when compared with histones isolated from the nuclear pellet (Fig. 4). However, whereas the ratio in acetylation of histone H3 between cell lysate versus nuclear pellet remained constant during the course of apoptosis, histone H4 was characterized by a progressive change in this ratio. Thus, whereas H4 retained in the nuclear pellet suffered a progressive decrease in acetylation, in agreement with the observations shown above, histone H4 in the cell lysate was more acetylated at later times after apoptosis induction.

The analysis of all the acetylatable positions of H4 (Lys-5, -8, -12, and -16) showed that released histone H4 was less acetylated in all cases than the H4 fraction retained in the nuclear pellet. In a time course experiment, acetylation of Lys-16 and Lys-12 followed a pattern similar to global acetylation and increased in the cell lysate fraction as apoptosis advanced, whereas no changes were observed in the acetylation of Lys-8 and Lys-5.

Mass spectrometry analysis of these samples (see supplemental Fig. S1A) confirmed that histone H4 of the apoptotic cell lysate was

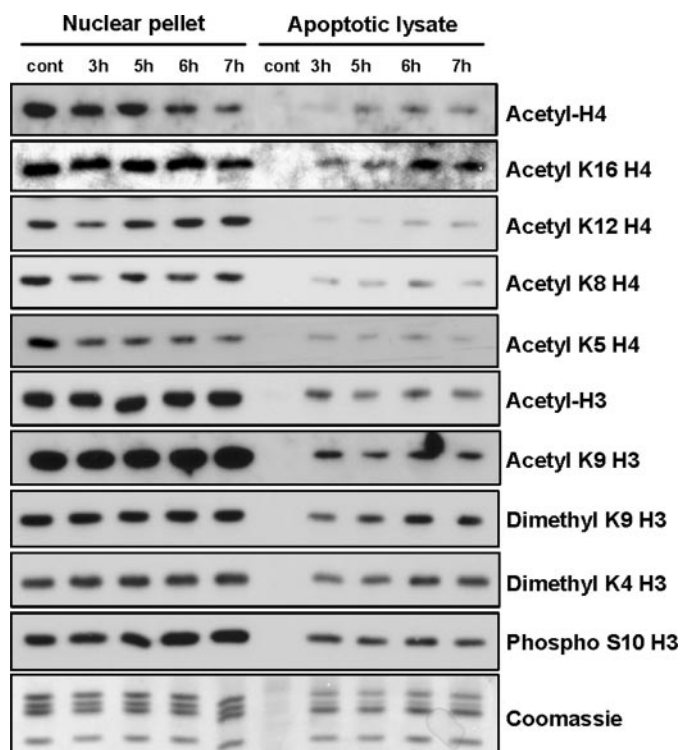


FIGURE 4. Western blot analysis of histone modifications of apoptotic cell lysate and nuclear pellet fractions. Analysis with antibodies against acetyl-H4 and acetyl Lys-16, -12, -8, and -5 H4 of histones extracted from HL60 cells at different times after apoptosis induction with etoposide. Coomassie staining is included as a loading control.

hypoacetylated relative to control and nuclear pellet samples (as already shown by Western blot and HPCE analysis). MS/MS analysis indicated that almost all detectable monoacetylation was found to occur in Lys-16 of histone H4 in all the samples. It can be observed that ion  $y_5$  (supplemental Fig. S1B), containing residues 13–17 (GGAKR) of histone H4, was almost completely acetylated in all samples, whereas ion  $b_9$ , containing residues 4–12 (GKGGKGLGK) of histone H4, was almost completely deacetylated in all samples (supplemental Fig. 1C) and there was no increase in acetylation of Lys-16 in cell lysates 3 h after treatment.

On the other hand, released histone H3 generally had a lower level of modification for all modifications studied; it was less acetylated, methylated, and phosphorylated than were histones isolated from the nuclear pellet. Whereas the levels of acetylated H3 remained constant, dimethylated forms of Lys-4 and Lys-9 exhibited a slight increase over time (Fig. 4).

*Internucleosomal Fragmented DNA Released in Apoptosis Is Hypermethylated with Respect to DNA That Remains in the Nucleus*—Histone methylation and acetylation have been shown to be mechanistically linked to DNA methylation (45). Therefore, the observed differences in histone methylation and acetylation during apoptosis and among fractions (apoptotic cell lysates and nuclear pellets) prompted us to investigate the existence of changes in DNA methylation during apoptosis. On the other hand, it is possible that changes in DNA methylation could participate in the apoptotic process, in a manner similar to those demonstrated in other processes, like cancer development (46, 47).

First, genomic DNA was obtained by a standard protocol from isolated nuclei of HL60 cells treated with etoposide at different times. The analysis of the 5-methylcytosine content of the samples showed a time-dependent decrease. The amount of total 5-methylcytosine genomic content decreased from 3.1% in control cells to 2.4% 6 h after apoptosis induction (Fig. 5A). These changes, although apparently small, occurred

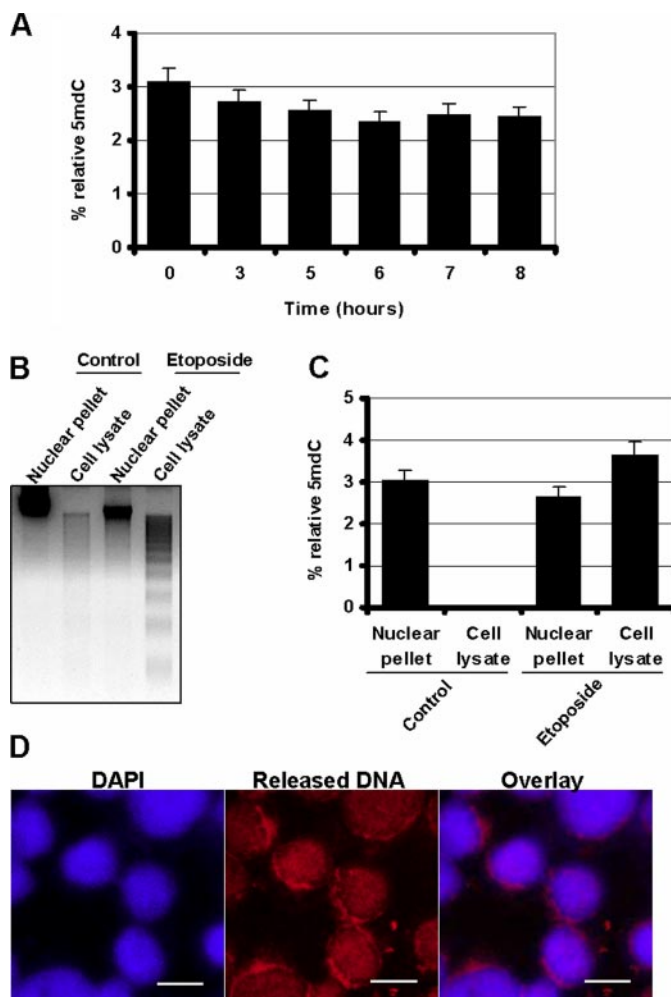


FIGURE 5. Analysis of DNA that cofractionates with histones released in apoptosis. A, analysis of 5-methylcytosine content during apoptosis in HL60 cells as quantified by HPCE. B, analysis by agarose electrophoresis of DNA isolated from the corresponding nuclear pellet and cell lysate samples of HL60 cells non-treated and treated for 8 h with etoposide. C, analysis of 5-methylcytosine content of DNA from nuclear pellet and cell lysate samples of control HL60 and at 3, 6, and 8 h after etoposide treatment. D, 4',6'-diamidino-2-phenylindole and nuclear-specific sequence localization of fragmented DNA isolated from the apoptotic cell lysate labeled with Spectrum Red in HL60 cells.

within the same range of changes experienced during tumoral processes (48, 49). The observed decrease in the 5-methylcytosine content of DNA isolated from nuclei could be explained if fractionation of the DNA between the nucleus and cytosol occurred during apoptosis, similar to the fractionation observed for histone modifications.

We therefore isolated DNA from the two fractions: nuclear pellet, which would account for the DNA isolated previously from the nuclei, and cell lysate, where the DNA released during apoptosis should be present. DNA was quantified and analyzed by agarose gel electrophoresis. Interestingly, we observed the characteristic apoptotic internucleosomal fragmentation pattern (together with large chromatin fragments of >5 kbp) only in the DNA isolated from the apoptotic cell lysate, whereas the DNA from the apoptotic nuclear pellet exhibited much less fragmentation (Fig. 5B).

DNA isolated from the apoptotic and control cell lysate fractions was then analyzed by HPCE to measure its 5-methylcytosine content. The data obtained showed enrichment in methylation in the apoptotic cell lysate, whereas there were no significant differences among the nuclear pellet samples (Fig. 5C). DNA corresponding to apoptotic cell lysates had a content of 5-methylcytosine of ~3.5% (Fig. 5C), which was signif-

## An Epigenetic Marker of Early Apoptosis

icantly higher than that of the cell line. This percentage is compatible with that found in chromatin regions with a high percentage of CpG dinucleotides, *i.e.* repetitive sequences and/or CpG islands of tumor-suppressor genes, which are methylated in HL60 cells. The hypermethylated status of released DNA, together with the hypoacetylation of the histones and trimethylation of H4 isolated in the same fraction, implies that the heterochromatin may be the source of the DNA and histones released during apoptosis.

**DNA Released during Apoptosis Comes from Perinuclear Regions Associated with Heterochromatin**—To investigate the origin of the nuclear material released during apoptosis, DNA isolated from the apoptotic cell lysate fraction was labeled with Spectrum Red and used as a probe to investigate the chromosomal origin of DNA released during apoptosis. In this case, Spectrum Red-labeled DNA from the cell lysate fraction was competitively hybridized against total DNA from HL60 cells in metaphase chromosomes. The presence of an even pattern of labeling throughout all the chromosomes (not shown) did not allow concluding that released DNA had a preferential chromosomal origin. We then used Spectrum Red-labeled released DNA in sequence-specific nuclear fluorescence localization in HL60 cells. This technique can be used to locate the nuclear origin of the fragmented DNA using a control non-treated cell as a target. The nuclear localization of the DNA associated with the apoptotic lysate revealed the formation of fluorescent rings at the nuclear periphery, indicating that the released DNA originated in the perinuclear region of the nucleus where chromatin interacts with the nuclear membrane mainly through heterochromatin sequences (25, 26, 50, 51) (Fig. 5D). This result is compatible with those obtained after the 5-methylcytosine analysis of the released DNA that had already indicated a possible heterochromatic origin.

### DISCUSSION

Histone modifications play key roles in different cellular processes, including replication, transcription, DNA repair, and chromatin condensation in mitosis (3). Several of the most recognizable events in apoptosis involve visible changes in the nucleus, including chromatin condensation, DNA fragmentation, and release of nuclear proteins (2, 20). It is very likely that histone modifications participate in these processes and probably coordinate and connect these events. Here we have demonstrated that changes in histone modifications do indeed occur in apoptotic cells, and we propose that a specific pattern of histone modifications existing before apoptosis could participate in events such as the release of nuclear material.

**Global Histone Hypoacetylation in Apoptosis**—Our results support the notion that global histone hypoacetylation occurs during apoptosis. Interestingly, hypoacetylation does not seem to be a passive apoptotic characteristic, because it is clearly associated with a fraction of the histones released from chromatin during its degradation. This observation directly associates histone hypoacetylation with the structural changes in chromatin taking place during apoptosis and its degradation.

It has been previously proposed (19) that global decrease of histone acetylation could be the result of a selective loss through proteolytic degradation in the cytosol of hyperacetylated histones released from euchromatin during early apoptosis and that this would cause a net increase in apparent global hypoacetylation. However, our results indicate that histones released during early apoptosis show significantly more hypoacetylation than do those remaining in the nucleus. A differential analysis of the apoptotic cell lysates, where the released histones are present, and nuclear pellets shows that global hypoacetylation mainly occurs in the released fraction, indicating that released histones come preferentially from hypoacetylated and not hyperacetylated chro-

matin. Although this experiment does not rule out the possibility of specific degradation of hyperacetylated histones, Western blot analyses of the samples from both cell lysate and nuclear pellet do not show significant degradation of histones H3 and H4 (Fig. 1A).

**Trimethylation of Histone H4 Is Specifically Associated with Histones Released during Apoptosis**—One of the most striking findings of this study is that the fraction of histone H4 released from chromatin in early apoptosis is exclusively trimethylated. This trimethylation has been previously demonstrated to occur at Lys-20 (40).

Although it is likely that H4 Lys-20 trimethylation acts as a passive feature or structural determinant for chromatin degradation and release, we cannot discount the possibility that it may play an active role as a recruiting signal for nuclear factors or nucleases in these processes. Thus, histone H4 trimethylation could act as a signal in a way similar to other post-translational modifications that recruit different nuclear complexes to chromatin (52, 53) in the context of the histone code (54). Trimethylation at Lys-20, alone or in combination with other modifications such as acetylation, could then act as a signal for enzymes that are capable of inducing DNA fragmentation and/or chromatin condensation, either directly in the case of nucleases that cleave DNA or indirectly if the recruited enzymes are associated with chromatin remodeling activities.

**Released Histones and DNA during Early Apoptosis Have Their Origin in the Perinuclear Heterochromatin**—The identity of the epigenetic marks and experiments to localize histones and DNA isolated from apoptotic cell lysates indicate that the material released from the nucleus in apoptosis has a perinuclear origin, at least during the initial stages. First, released DNA isolated from apoptotic cell lysates is hypermethylated and, when labeled and used as a probe for staining, exhibits perinuclear localization. On the other hand, released histones are hypoacetylated and histone H4 is trimethylated at Lys-20, a characteristic epigenetic mark of constitutive heterochromatin (8, 9, 41, 43, 55). In fact, we have previously demonstrated that trimethylated histone H4 at Lys-20 is associated with satellite and other repetitive sequences (40) that are known to be part of constitutive heterochromatin.

Internal nuclear structures become degraded before apoptotic chromatin condensation and DNA fragmentation, (22–24, 28). These include heterochromatin structures by which chromatin is anchored to the nuclear membrane (25, 26, 51, 56). This is even stronger evidence in support of the hypothesis that histone H4 and fragmented DNA released and isolated in the cell lysate fraction are a direct consequence of the perinuclear heterochromatin degradation described.

It has been proposed that chromatin condensation during apoptosis consists of a process of several consecutive steps. In the first step, endonuclease-hypersensitive euchromatin, which is hyperacetylated, would be degraded, followed or accompanied by the degradation of both the nuclear lamina and components of the intranuclear protein matrix during early apoptosis. The collapse and aggregation of heterochromatin would follow, giving rise to the characteristic apoptotic chromatin condensation. Afterward, there would be a second degradation process of the heterochromatin that would yield the characteristic oligonucleosomal DNA ladder (19). Therefore, should this model be correct, the majority of the apoptotic cell lysate histones and DNA would be the product of the heterochromatin collapse and degradation process, most probably from the perinuclear heterochromatin region degraded along the nuclear lamina. We have not been able to identify the presence of hyperacetylated histones in the apoptotic cell lysate during early apoptosis prior to the hypoacetylated and trimethylated histone H4 release. Therefore, this model implies that no histones or DNA are released

from euchromatin degradation even if this occurs before heterochromatin collapse and degradation takes place.

The unique content of epigenetic modifications in histone and DNA released during apoptosis and characterized by the methylation and acetylation status of histone H4 from perinuclear heterochromatin and hypermethylation of DNA suggests that these modifications may provide cells with the adequate context, perhaps by the formation of specific nuclear structures, specific substrates, or both, to initiate the degradation of the nucleus in apoptosis and to control chromatin condensation.

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