Modeling Apoptotic Chromatin Condensation

in Normal Cell Nuclei:

Requirement for Intranuclear Mobility and Actin Involvement*

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FOOTNOTES

* This work was supported in part by Grant 6P04A01317 from the Polish Committee for Scientific Research KBN (to P. W.), Grant GM01-59809 from the National Institutes of Health, and Grant I-0823 from the Robert A. Welch Foundation (to W. T. G.).

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The abbreviations used are: AIF, apoptosis-inducing factor; AMP-PNP, 5'-adenylylimidodiphosphate; BLM, bleomycin; CAD, caspase-activated deoxyribonuclease; CPAN, caspase-activated nuclease; DFF, DNA fragmentation factor; ICAD, inhibitor of CAD; m-AMSA, 4’-(9-acridinylamino)methanesulfon-m-aniside; MNase, micrococcal nuclease; topo II, topoisomerase II; VM-26, teniposide.
ABSTRACT

Hallmarks of the terminal stages of apoptosis are genomic DNA fragmentation and chromatin condensation. Here we study the mechanism of condensation both in vitro and in vivo. We find that DNA fragmentation per se of isolated nuclei from non-apoptotic cells induces chromatin condensation that closely resembles the morphology seen in apoptotic cells, independent of ATP utilization, under physiological ionic strengths. Interestingly, chromatin condensation is accompanied by release of nuclear actin, and both condensation and actin release can be blocked by reversibly pre-treating nuclei with either Ca^{2+}, Cu^{2+}, diamide, or low pH, procedures shown to stabilize internal nuclear components. Moreover, specific inhibition of nuclear F-actin depolymerization or promoting its formation also reduces chromatin condensation. Chromatin condensation can also be inhibited by exposing nuclei to reagents that bind to the DNA minor groove, which disrupt native nucleosomal DNA wrapping. In addition, in cultured cells undergoing apoptosis, drugs that inhibit depolymerization of actin or bind to the minor groove also reduce chromatin condensation but not DNA fragmentation. Therefore, the ability of chromatin fragments with intact nucleosomes to form large clumps of condensed chromatin during apoptosis requires the apparent disassembly of internal nuclear structures that may normally constrain chromosome subdomains in non-apoptotic cells.
INTRODUCTION

Characteristics of the terminal stages of apoptosis are genomic DNA fragmentation and chromatin condensation (reviewed in Ref. 1). Although internucleosomal DNA breakdown is often temporally correlated with such chromatin condensation, it is not absolutely required to trigger this event (2-4). Three pathways have been identified that mediate apoptotic chromatin condensation: (i) A caspase-3 independent pathway triggered by mitochondrial AIF, which leads to an accompanying large-scale DNA fragmentation without internucleosomal DNA cleavage (5); (ii) A caspase-3 dependent pathway triggered by the protein acinus, which occurs without inducing any DNA fragmentation (6); and (iii) A caspase-3 dependent pathway that leads to internucleosomal DNA cleavage mediated by activated DFF, also termed CAD or CPAN (7-11).

Although little is known regarding the mechanisms that lead to chromatin condensation in apoptotic cells, considerable information has appeared in the literature on the components that are linked to chromatin condensation in non-apoptotic cells. In interphase nuclei, heterochromatic regions are often associated with specific chromosomal proteins, post-translational modifications, and methylated DNA (reviewed in Refs. 12-14). In mitotic cells, chromosome condensation requires post-translational modifications and the action of an ATP-dependent complex called “condensin” to introduce positive DNA supercoils into DNA substrates in the presence of topoisomerases (15; reviewed in ref. 16). In summary, many parameters and protein factors have been associated with the formation of heterochromatin and condensation of mitotic chromosomes, but which, if any of these apply to chromatin condensation during apoptosis remains to be determined.

Here we investigate the mechanism of chromatin condensation mediated by the internucleosomal DNA cleavage pathway in apoptosis. Previously we demonstrated that purified activated recombinant DFF triggered chromatin condensation when added to nuclei isolated from non-apoptotic cells (8). In the present study we have exploited this observation to dissect the mechanism of condensation triggered by internucleosomal DNA fragmentation using the well
established model of isolated HeLa cell nuclei for \textit{in vitro} reconstitution experiments of apoptotic events (7, 17). Where possible we have complemented our \textit{in vitro} findings by utilizing cultured human leukemic HL-60 cells triggered to undergo apoptosis with etoposide or cisplatin (18). Our results reveal that chromatin condensation under physiological ionic strength requires intranuclear mobility, which is allowed by the apparent disruption of components that may compartmentalize chromosome subdomains in normal cells. In addition, such condensation requires intact nucleosomes.

**EXPERIMENTAL PROCEDURES**

\textit{Cell culture and Treatment.} Human lymphoblastoid HL-60 cells, cultured in RPMI 1640 medium supplemented with 10\% fetal bovine serum, were exposed to etoposide (Bristol-Myers) or cisplatin (Ebewe) at final concentrations of 34 and 27 $\mu$M, for 6 and 12 h, respectively. In some experiments, DNA minor groove-binding drugs or actin-binding drugs (at concentrations indicated in text) were added to medium 30 min prior to etoposide. Cells were then washed with PBS buffer, fixed with 10\% formaldehyde for 1 h at 4°C, washed with PBS and then transferred onto microscope slides, dried and stained with 10 $\mu$M DAPI. DNA from portions of PBS-washed cells was purified and analyzed by agarose gel electrophoresis as described below. For labeling replicating DNA, 200 $\mu$M each of BrdU and deoxycytidine were added to the above media and after 10 h cells were pelleted, resuspended in fresh media and treated with drugs as above for 6 h. Samples that were fixed with 10\% formaldehyde for 10 min were used for detection of BrdU-labeled DNA as described elsewhere (19). Briefly, after washing fixed cells with PBS, treatment with methanol/acetic acid (3:1) and transfer onto slides, DNA was denatured, reacted with mouse
anti-BrdU monoclonal antibody (Sigma) and visualized by goat anti-mouse Ig antibody conjugated with Texas Red (Jackson ImmunoResearch Laboratory). For nuclease treatment \textit{in situ} HL-60 cells were permeabilized with lysolecithin as described elsewhere (20). Briefly, \(2 \times 10^6\) PBS-washed-cells were incubated for 1 min at 37°C with 0.05% lysolecithin in incubation buffer [0.1 M KCl, 0.15 M sucrose, 4 mM MgCl\(_2\), 1 mM CaCl\(_2\) and 20 mM Hepes, pH 7.5]. Cells were then washed once in incubation buffer by gentle centrifugation and suspended in 0.2 ml isolation buffer supplemented with 20 units of MNase. After 20 min incubation at 34°C cells were fixed with 10% formaldehyde.

\textit{Isolation and Treatment of Nuclei.} Nuclei were purified from HeLa S3 or HL-60 cells. Cells were washed by centrifugation in PBS buffer at 4°C and suspended in cold isolation buffer \([10 \text{ mM KCl, 0.25 M sucrose, 4 mM MgCl}_2, 1 \text{ mM DTT, 20 mM Hepes, pH 7.5 and Complete}^{\text{TM}} \text{(Roche) proteases' inhibitors set}].\) NP-40 was added to 0.5% and cells were incubated 10 min on ice. After washing twice with isolation buffer by centrifugation, nuclei were suspended in isolation buffer with 50\% glycerol and stored at \(-80\)°C or used fresh. The purified nuclei (\(\sim 30 \mu\text{g of protein}) were incubated with activated DFF (100 U/ml), MNase (50 U/ml, Worthington) or DNase I (10 U/ml, Roche) for 30 min at 34°C in 50 µl of buffer consisting of 100 mM KCl, 4 mM MgCl\(_2\), 1 mM CaCl\(_2\), 0.5 mM ATP, 1 mM DTT and 20 mM Hepes, pH 7.5. Human recombinant DFF was purified from an \textit{Escherichia coli} expression system and activated by incubation with caspase-3 as described previously (21), and then caspase was inhibited by 10 µM DEVD. In some experiments nuclei were incubated with \textit{Alu}I enzyme (1000 U/ml, Roche) or bleomycin (1 mg/ml, Nippon Kayaku). When indicated, nuclei were pre-treated with apyrase (10 U/ml for 15 min at 34°C) or ATP was replaced with ATP\(_\gamma\)S or AMP-PNP, or nuclei were treated with
nucleases in the presence of 1 mM ZnCl₂ or 10 mM KCl. In some experiments, immediately before treatment with nucleases, nuclei were incubated for 30 min at 34°C with following compounds (at concentrations indicated in Figures Legends): distamycin A, chromomycin A₃, bisbenzimide (Hoechst 33258), methyl green, ethidium bromide, etoposide, phalloidin, latrunculin A or jasplakinolide (last two from Molecular Probes, former from Sigma).

Extraction of Nuclei and Stabilization of Intranuclear Components. HeLa nuclei were extracted with 0.2- or 0.35-M NaCl in a buffer consisting of 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 20 mM Hepes, pH 7.5. Nuclei were incubated for 30 min on ice and then centrifuged for 15 min at 400Xg (control nuclei were incubated in the same buffer but containing 50 mM NaCl). HeLa nuclei were treated with acidic buffer according to the procedure of Lawson and Cole (22). Nuclei were suspended in 68 mM citric acid-sodium phosphate buffer at pH 4.0 containing 25 mM KCl, 0.25 M sucrose, 5 mM MgCl₂, and then incubated on ice for 30 min. Nuclei were centrifuged for 15 min at 400Xg and then washed twice with neutralization buffer (25 mM KCl, 0.25 M sucrose, 5 mM MgCl₂ and 20 mM Hepes, pH 7.5). HeLa nuclei were incubated on ice with either 5 mM CuCl₂ (pre-treatment of nuclei with Cu²⁺ diluted to 1 mM was equally effective in blocking condensation), or 10 mM diamide in neutralization buffer, or in 25 mM CaCl₂ in neutralization buffer at 37°C for 15 min, and then washed twice with cold neutralization buffer.

Analysis of DNA Laddering and Chromatin Condensation. After incubation of nuclei with nucleases, one-fourth of the reaction mixture was subjected to DNA analyses by agarose gel electrophoresis as described previously (21). The rest of the reaction mixture was fixed with 10% formaldehyde for 1 h on ice. Nuclei were then centrifuged and suspended in 10 µM DAPI dissolved in the reaction mixture buffer. Samples were visualized by epifluorescence microscopy.
and images acquired with a color CCD camera. Numbers below panels show the percentage of nuclei exhibiting chromatin condensation (average of 2-3 independent experiments with 200-300 purified nuclei or 500-1000 cells being scored per experiment).

Protein Analyses. Proteins from nuclei or nuclear extracts were separated on 15% polyacrylamide/SDS gels, and then stained with Coomassie Brilliant Blue or electrophoretically transferred onto nitrocellulose membranes (Protran, Schleicher and Schuell). Membrane-immobilized proteins were probed with mouse anti-human actin monoclonal antibody and visualized with goat anti-mouse Ig antibody conjugated with horseradish peroxidase (Oncogene Research Products).
RESULTS

*Internucleosomal DNA Fragmentation of Nuclei Isolated from Non-apoptotic Cells Induces Chromatin Condensation Resembling that Seen in Apoptotic Cells* - We first directly compared the morphology of chromatin condensed in cultured cells undergoing apoptosis with that triggered by DNA fragmentation *in vitro* in nuclei isolated from the corresponding non-apoptotic cells. For this purpose we treated cultured human HL-60 cells with either etoposide or cisplatin to induce apoptosis and pair-wise compared the extent of nucleosomal DNA laddering with chromatin condensation in these samples, and likewise in nuclei isolated from untreated HL-60 cells (Fig. 1). In agreement with our previous results (8), the morphology of nuclei in cells undergoing apoptosis was similar to that induced in nuclei isolated from normal cells after DFF treatment, as visualized by fluorescent microscopy after DNA staining with DAPI (Fig. 1B). In the context of the work reported here, we will refer to this morphology as that representing “chromatin condensation”, which we define as the coalescence of chromatin into distinct clumps localized mostly at the nuclear periphery. However, such condensation was not DFF-specific, because DNA fragmentation either by micrococcal nuclease (MNase), DNase I, *Alu*I, or even the radiomimetic antibiotic bleomycin (BLM) (reviewed in Ref. 23), also led to chromatin condensation (Fig. 1B). We have also found in this *in vitro* system that the extent of condensation correlated with the degree of DNA fragmentation and that condensation could be observed within 5 min if high levels of nuclease were used; endonuclease G, another protein involved in apoptotic DNA breakdown (24), was also effective at inducing chromatin condensation (data not shown). Chromatin condensation could also be induced in nuclei of cells treated with nuclease *in situ* after cytoplasmic membrane perforation (Fig. 1C). Differences in the morphology of condensation
between apoptotic cells (Fig. 1B, subpanels 2,3) and nuclease-treated nuclei or cells (Fig. 1B, subpanels 5-9; Fig. 1C, subpanel 2) are evident, including a lower background of DNA staining between condensed chromatin clusters and more irregular shaped nuclear envelopes in the apoptotic cells. Nevertheless, in response to DNA breakdown per se in this in vitro system, it is significant that the morphology of chromatin condensation closely resembles peripheral chromatin condensation visible in apoptotic cells [late stage I or early stage II as defined by Refs. (25, 26)].

**Chromatin Condensation is Energy Independent and Requires Physiological Concentrations of Mono- and Divalent Cations** - Mitotic chromosome condensation requires ATP hydrolysis (15), and an ATP requirement has been found for certain apoptotic cell extracts to trigger chromatin condensation (27). We therefore investigated the energy requirements for condensation in nuclei isolated from non-apoptotic HeLa cells. Nucleosomal DNA laddering mediated by either DFF or MNase was independent of the presence of ATP, ATPγS or apyrase (Fig. 2A, lanes 3-6 and 8-11) but was blocked by 1 mM ZnCl₂ (Fig. 2A, lanes 2,7), a known potent inhibitor of nucleases (11, 21). In addition, varied KCl concentrations slightly modulated such laddering (low [KCl] stimulated MNase but decreased DFF activity) (Fig. 2C). Chromatin condensation in these same samples was manifested only when DNA laddering had occurred and was not inhibited by ATPγS, apyrase (Fig. 2A,B), or AMP-PNP (data not shown). Furthermore, condensation mediated either by DFF or MNase was less evident at lower KCl concentrations (Fig. 2D, compare subpanels 1 and 2, and 3 and 4) and could be largely reversed by addition of 10 mM EDTA to digested nuclei (Fig. 2D, lower subpanels). Condensation could also be induced upon shifting MNase-digested nuclei from 10- to 100-mM KCl (data not shown). The observed
reversibility of condensation to changes in the concentrations of mono- and divalent ions is not unexpected, because previous studies have shown that even isolated mononucleosomes bearing histone H1 can be quantitatively precipitated by exposure to either 100 mM KCl or 5 mM MgCl₂, and subsequently reversibly quantitatively solubilized by exposure to low ionic strengths and EDTA (28, 29). Nevertheless, we conclude that coalescing of fragmented chromatin into condensed clumps is energy independent and optimal under physiological ionic strengths.

**Chromatin Condensation is Blocked by Stabilization of Internal Nuclear Components**- In considering the mechanism of chromatin condensation in our *in vitro* system, it seems clear that digested chromatin fragments need to be able to diffuse within isolated nuclei in order to coalesce into clumps of condensed chromatin. However, nuclei within living cells are known to be organized into subcompartments, which have established territories for individual chromosomes and limit chromatin segment movement to nuclear subregions (30; reviewed in Refs. 12, 31-33). Possibly this internal nuclear organization becomes altered during the processes of cell lysis and nuclei isolation such that intranuclear diffusion of chromatin fragments becomes possible. Indeed, during apoptosis in cultured cells the accompanying proteolysis of nuclear lamins and other nuclear proteins leads to disorganization of nuclear subcompartments (25, 34, 35).

To test the idea that dissolution of internal nuclear structure is required for chromatin condensation in our *in vitro* system, we took advantage of the observations made by Laemmli and co-workers that reversible treatment with Ca²⁺ or Cu²⁺ stabilize DNA attachment sites both in HeLa cell mitotic chromosomes and interphase nuclei (36-38) (reviewed in Refs. 39-41). Indeed, it has recently been demonstrated that cellular chromatin *in vivo* possesses unexpectedly high
concentrations of Ca\(^{2+}\) (4-9 mM for interphase nuclei, and 20-32 mM for mitotic chromosomes), which are at 3-fold higher levels than those of Mg\(^{2+}\) ions; furthermore, such Ca\(^{2+}\) is enriched in the chromosomal axis and co-localizes with the scaffolding proteins topo II and ScII (42). We also used two other techniques to stabilize internal nuclear components, exposure of nuclei to mild oxidation to generate disulfide bonds within and between protein species (reviewed by in Refs. 39-41), and exposure of nuclei to pH 4.0 followed by neutralization to pH 7.5 (our empirical observation). As shown in Fig. 3A, these pre-treatments did not significantly disrupt chromatin structure as revealed by the patterns of DNA laddering generated by subsequent digestion with MNase (Fig. 3A, lanes 2-5) or DNase I (data not shown). However, such pre-treatments severely inhibited subsequent chromatin condensation (Fig. 3B, subpanels 2-5). (For these and further experiments for convenience we have primarily employed MNase as the nuclease used to trigger condensation). In addition, chromatin condensation in control nuclei could not be reversed if any of these treatments occurred after nuclease digestion (data not shown).

Significantly, all pre-treatments that blocked chromatin condensation in isolated nuclei affected the extractability of nuclear proteins by 0.2- and 0.35-M NaCl. Either reversible treatment with CaCl\(_2\), diamide or low pH significantly reduced the amount of released protein, while CuCl\(_2\) pretreatment almost totally prevented nuclear protein release (Fig. 3C). In conclusion, enhancing metalloprotein interactions, inducing disulfide bond formation, and apparent protein denaturation caused by reversible low pH treatment each apparently stabilize internal nuclear components and thereby disallow digested chromatin fragments the intranuclear mobility required for coalescence into clumps of condensed chromatin.
Evidence for Nuclear Actin Involvement in Chromatin Condensation- Actin is among the candidate filament proteins that may participate in nuclear subcompartmentalization (reviewed in Ref. 41). To test directly for an actin involvement in chromatin condensation, we employed the drugs latrunculin A and phalloidin, which respectively depolymerize or polymerize actin filaments (F-actin) (43, 44). As shown in Fig. 4A, treatment of nuclei with these reagents did not significantly disrupt chromatin structure as revealed by the patterns of DNA laddering. Actin is a known inhibitor of DNase I, which depolymerizes F-actin (45). Significantly, treatment of nuclei with phalloidin markedly increased the sensitivity of chromatin to DNase I digestion (Fig. 4A); reversible exposure of nuclei to pH 4 (but not pre-treatment with diamide, CaCl₂ or CuCl₂) also sensitized nuclei to DNase I digestion (data not shown). Furthermore, phalloidin treatment partially inhibited chromatin condensation (Fig. 4B, subpanel 2). It is known that phalloidin will cause the formation of F-actin from G-actin (44). However, the partial inhibition caused by phalloidin was apparently not due to causing this conversion because a similar extent of inhibition was observed when latrunculin A was added at the same time to block F-actin formation (Fig. 4B, subpanel 4). Although such inhibition was detected in only ~30% of the nuclei, it proved to be highly reproducible and statistically significant (P < 0.05). In addition, use of jasplakinolide, another reagent known to stabilize F-actin (46), gave the same degree of partial inhibition of condensation (data not shown). Furthermore, chromatin condensation in control nuclei could not be reversed if the phalloidin treatment occurred after nuclease digestion (data not shown). Interestingly, if the nuclei were first extracted with 0.35 M salt, a condition leading to ~60% loss of nuclear actin, chromatin condensation could no longer be partially inhibited by phalloidin (data not shown). We also analyzed nuclear proteins released from MNase-digested nuclei by SDS-PAGE and actin by Western blotting (Fig. 4C,D). Concomitant with the
condensation process we found that >80% of the nuclear actin was lost from control nuclei, or nuclei pre-treated with latrunculin A (Fig. 4C,D, lanes 1,2,5,6). Significantly, such a loss was blocked by reversible exposure to pH 4 (Fig. 4D, lanes 9,10), as well as by pre-treatment with CaCl₂ (Fig. 4D, lanes 7,8), CuCl₂ (Fig. 4D, lanes 11,12) or diamide (Fig. 4D, lanes 13,14). Interestingly, phalloidin treatment only partially blocked actin release (Fig. 4C,D, lanes 3,4). This correlates with the observation that phalloidin treatment only fractionally inhibited condensation, unlike pH 4, CuCl₂, or diamide. Taken together, these results suggest that F-actin partially restricts chromatin fragment mobility for condensation.

We also assayed actin-targeting reagents for their ability to block chromatin condensation in cultured HL-60 cells triggered to undergo apoptosis by etoposide. We selected jasplakinolide to stabilize F-actin because it is much more permeable to cells than phalloidin (46), and latrunculin A which targets G-actin (43). Because we found that the drugs were quite toxic to cells we had to perform experiments with reagents diluted to 0.3 µM. Under these conditions we found that jasplakinolide (±latrunculin A) treatment did not affect DNA laddering induced by etoposide (Fig. 4E, lane 5,6), whereas these drugs reduced chromatin condensation about 20% compared to the etoposide-treated control (70% and 69% vs. 89%) (Fig. 4F, subpanels 4,5,6). Jasplakinolide by itself also induced minor DNA breakdown (Fig. 4E, lane 2), which is in agreement with a previous report that the reagent enhances apoptosis (47). Nevertheless, we have evidence both in vitro and in vivo that F-actin apparently partially restricts chromatin fragment mobility for condensation.
The observed partial inhibition of chromatin condensation by drugs that stabilize F-actin may be related to cell cycle events that modulate nuclear actin (levels, post-translational modifications, organization and/or associated proteins). One might expect that nuclei of S phase cells may be less restrictive to chromatin mobility because this period corresponds to the time when chromosomal domains must become repositioned to immobile replication centers for their subsequent DNA replication (48, 49). Therefore we analyzed the extent of chromatin condensation in nuclei of cells containing newly replicated DNA. HL60 cells growing at log-phase with a ~25 hr doubling time were pulse-labeled with BrdU for 10 hr immediately before treatment with etoposide and jasplakinolide during the chase. Under these conditions about 35% of the cells had incorporated BrdU. Significantly, in this cell population ~50% of the nuclei with condensed chromatin contained newly synthesized DNA, while only ~5% of the cells that lacked condensed chromatin were labeled with BrdU (Fig. 4G). Therefore, stabilization of F-actin is primarily inhibitory of apoptotic chromatin condensation in non-S-phase cells.

Agents that Target the DNA Minor Groove Block Chromatin Condensation

In order to dissect further the mechanism of chromatin condensation in our in vitro system we searched empirically for other reagents that could uncouple condensation from DNA fragmentation at physiological ionic strengths. We selected for study agents that bind to or intercalate into either the minor or major grooves of DNA, including: distamycin A and bisbenzimide (Hoechst 33258), which preferentially bind to the minor groove of AT-rich DNA (50, 51); chromomycin A₃, which preferentially binds to the minor groove of GC-rich DNA (52, 53); ethidium bromide, which intercalates into the DNA minor groove (54); and methyl green, which binds to the DNA major groove (55).
As shown in Fig. 5, several of these reagents blocked condensation without inhibiting DNA fragmentation. With the exception of ethidium bromide (Fig. 5A, lane 7), incubation of nuclei with any of the DNA-binding compounds did not markedly disrupt chromatin structure as revealed by the patterns of DNA laddering on a low resolution agarose gel (Fig. 5A). Because ethidium bromide is known to perturb chromatin structure (56), this result was not unexpected. Both distamycin A and bisbenzimide, but not chromomycin A₃ or ethidium bromide, however, affected the rate of digestion (Fig. 5A). Significantly, distamycin A, chromomycin A₃, bisbenzimide and ethidium bromide, all minor groove targeting drugs, each severely blocked chromatin condensation (Fig. 5B, subpanels 3,4,5,7). In marked contrast, incubation with the major groove binder, methyl green, or with the topo II inhibitors etoposide, VM-26 or m-AMSA, did not inhibit subsequent condensation (Fig. 5B, subpanels 6,8; data not shown). Furthermore, addition of minor groove reagents after DNA fragmentation did not reverse chromatin condensation (Fig. 5B, subpanels 9-11), whereas chromatin condensation was still inhibited by DNA binding drugs if nuclei were first extracted with 0.35 M NaCl (data not shown). We conclude that interactions which involve the DNA minor groove are fundamentally required to trigger chromatin condensation in our in vitro system, regardless of DNA sequence preferences.

Analysis of the dose response of the inhibitory reagents reveals that 50% inhibition of condensation occurs between 20- and 50-µM drug (Fig. 5C). We have quantitated spectrophotometrically the amount of these reagents that bind to nuclei at 50% inhibition, and estimate that if all reagent that is bound to nuclei is associated with DNA that 1 molecule of drug would exist for every 10-20 bp. One would expect that DNA wrapping about and within
nucleosomes might be disrupted at these levels of drug, although we did not see any disruption of the nucleosomal repeat on a low resolution gel, with the exception of ethidium bromide treatment (Fig. 5A). However, analysis of digestion products on a higher resolution acrylamide gel reveals that the 146 bp barrier, which is a characteristic of the nucleosome core particle (57), is lost at drug concentrations that block condensation, and there is a higher internucleosomal band background, indicating that the drugs increase nuclease cleavage within nucleosomes (Fig. 5D).

To examine alterations in DNA wrapping caused by these reagents in more detail, we assayed for the intactness of the ~10 base subnucleosomal DNA repeat that is generated by DNase I digestion of chromatin (57, 58). As shown in Fig. 5E, this subnucleosomal DNA repeat is also disrupted by the inhibitory drugs, as evidenced by a higher interband background. By contrast, no detectable disruption in nucleosomal DNA wrapping was observed at even 100 µM methyl green, a concentration which we estimate from what bound to nuclei would represent roughly 1 molecule of drug for every 20 bp (Fig. 5E). Although similar concentrations of minor groove drugs were toxic to HL-60 cells, at lower doses they were partially inhibitory to etoposide-induced apoptotic condensation (data not shown). We conclude that intact nucleosomes are required for chromatin condensation in our system.
DISCUSSION

Conflicting reports exist in the literature on whether DNA cleavage of nuclei isolated from non-apoptotic cells by any nuclease can lead to chromatin condensation nearly mimicking that seen in apoptotic cells (2, 5, 33, 59, 60). If this indeed were true, then a model *in vitro* system could be established to dissect the requirements for this process. Here we have successfully developed such a system. We provide also an explanation for discrepancies in the literature because we demonstrate that such condensation requires mono- and divalent metal ions at physiological ionic strengths; conditions not always used by other investigators. It should also be noted that the nature of DNA fragment ends is not important, as condensation can occur with components possessing blunt ends, with 5’ or 3’ overhangs, and with either 5’ or 3’ phosphate groups.

Condensation Apparently Requires Disruption of Nuclear Sub-compartmentalization- We have evidence that chromatin condensation in our *in vitro* system requires intranuclear mobility mediated by the disruption of components that may participate in the subcompartmentalization of chromosomal subdomains in living cells. We found that enhancing metalloprotein interactions, inducing disulfide bond formation, or apparent protein denaturation caused by reversible low pH treatment each were effective in blocking chromatin condensation. These treatments inhibit condensation by a mechanism different from the drugs that target the DNA minor groove because we have found that the subnucleosomal DNase I-generated ladder remains intact in such treated nuclei (data not shown). In only one of these four conditions (low pH), pre-extraction of nuclei with 0.35 M NaCl eliminated the block in apparent intranuclear mobility (data not shown).

While it can be argued that these stabilization treatments are quite harsh and may lead to artifacts, stabilization of nuclear F-actin led to partial inhibition of chromatin condensation, both *in vitro* and *in vivo*, strongly supporting the notion that disruption of intranuclear organization is indeed required for chromatin condensation. Furthermore, leakage of nuclear actin was markedly reduced, either by pH 4, Ca²⁺, Cu²⁺ or diamide pre-treatments, conditions that each severely
inhibited chromatin condensation. Interestingly, previous micro-dissection experiments with *Xenopus* oocytes have revealed that even after mechanical removal of the nuclear envelope, chromosome organization is maintained by a “nucleoplasmic gel” that contains both F- and G-actin as major components (61, 62). Indeed, it is now appreciated that nuclear actin plays roles in chromatin remodeling and RNA transcript trafficking (63, 64). Furthermore, certain actin-binding proteins interact with a fibrous network in the nucleoplasm (64, 65). We propose that nuclear actin also participates in establishing chromosomal territories that restrict chromatin fragment mobility. Stabilization of F-actin, however, was ineffective in blocking chromatin condensation in cells replicating their genomes. This suggests that the apparent intranuclear mobility of chromatin is less restricted by F-actin during S phase of the cell cycle. It is of further interest that actin is a known substrate for caspase-3 during apoptosis (66).

**Condensation Likely Requires Preservation of Native Nucleosomal DNA Wrapping through Core Histone:DNA Minor Groove Contacts** - It is striking that agents that target the minor groove of DNA inhibit chromatin condensation, both *in vitro* and *in vivo*. These agents inhibit condensation by a pathway not linked to actin retention (data not shown). Distamycin A and bisbenzimide preferentially bind to the minor groove of AT-rich DNA, eliminate the known preferential association of histone H1 with AT-rich DNA (67, 68) and cause decondensation of heterochromatin (69, 70). However, chromomycin A₃, which preferentially binds to the minor groove of GC-rich DNA was also effective in blocking condensation. We have searched by SDS-PAGE for proteins that might be released from nuclei by ethidium, distamycin A or chromomycin A₃ treatment, but found no evidence for release or weakened interactions with DNA of any protein species under conditions that blocked condensation (data not shown). Nuclease
protection assays however, have revealed that these inhibitory drugs alter nucleosomal DNA wrapping. The globular domain of histone H1 binds to the major groove of DNA (71, 72), whereas core histones bind to the minor groove (73). Native nucleosomal DNA wrapping through core histone interactions therefore seems to be required for nucleosomes to assemble into higher order structures that lead to chromatin condensation. This wrapping is known to neutralize positively-charged amino acids by a close association with negatively-charged DNA phosphate groups. However, simple electrostatic repulsion between chromatin fragments can not be the mechanism behind the inhibition of condensation mediated by these drugs, because neither distamycin A nor ethidium bromide (at up to 200 µM concentrations) prevented precipitation of isolated oligonucleosomes (1 to 8-mers) induced by 4 mM MgCl₂ and 100 mM KCl (data not shown). Thus, it appears that these drugs affect the ability of chromatin fragments to coalesce, possibly by promoting interactions between the drug-induced exposed positively-charged amino acids and acidic non-histone proteins, which may interfere with inter-nucleosome interactions and/or fragment diffusion. It is interesting to reflect from an evolutionary view that nucleosome structure not only fulfills crucial regulatory and packaging roles in living cells, but also prepares apoptotic cells for the efficient clearance of DNA by phagocytosis during cell death. Although not known at present, we favor the notion that the condensation process that we observe represents a specific form(s) of stacking of nucleosomes into supramolecular structures, rather than non-specific aggregation phenomena. Indeed, it has been recently shown that at high concentrations of nucleosome core particles (350 mg/ml), which is similar to their intranuclear level, nucleosomes stack into aligned columns that form bilayers constituting a liquid crystalline state (74).
Extensive earlier investigations have demonstrated that histone H1 facilitates higher-order chromatin packing (75; reviewed in Ref. 76), and confers insolubility even to mononucleosomes at physiological ionic strengths (28, 29). The observed ionic strength dependency on chromatin condensation is consistent with a role for histone H1 in this process. Furthermore, it is known that histone H1-minus-nucleosomes leak out of MNase-digested-nuclei under physiological ionic strengths (77). To determine if condensation could be inhibited by depleting nuclei of histone H1 prior to DNA fragmentation, we extracted nuclei either at low pH (22), with polyglutamic acid (68), or with 0.6 M NaCl. However, in our hands we found that these procedures were either inefficient or disruptive to nuclear integrity (data not shown).

*Neither Topo II Activity nor ATP are Required for Condensation-* Distamycin A is also known to inhibit the preferential interaction of topo II with AT-rich DNA (78). Topo II has been associated with mitotic chromosome condensation in normal cells (79-81), with heterochromatin formation in interphase nuclei (82), with mediating DNA aggregation *in vitro* (78, 83), with chromatin condensation triggered by apoptotic cell extracts (84), and with higher-order DNA fragmentation during apoptosis (85). In addition, previous studies have shown that ATP is required for chromatin condensation but not for internucleosomal DNA fragmentation when apoptotic cell extracts are added to isolated nuclei from non-apoptotic cells (27). However, inhibition of topo II activity or depletion of ATP were ineffective in blocking chromatin condensation in our experiments with isolated nuclei. Therefore we suggest that in these earlier studies the topo II and ATP requirements may play a role in eliminating inhibitors present in apoptotic cell extracts.
Acknowledgements- We thank Sveta Earnst, Michael Hale, Katherine Meyers and Ying Zou for help in this work.
REFERENCES

FIGURE LEGENDS

FIG. 1. Comparison of DNA fragmentation and chromatin condensation patterns between HL-60 cells undergoing apoptosis and nuclei purified from non-apoptotic cells. Panel A - DNA from cells exposed in culture to etoposide or cisplatin, or from isolated nuclei incubated with either activated DFF, MNase, DNase I, AluI, or bleomycin (BLM), was analyzed by 1.5% agarose gel electrophoresis. Panel B - Micrograph showing morphology of nuclei from apoptotic cells or purified nuclei treated with nucleases or BLM in vitro. Panel C – Analysis of DNA fragmentation and morphology of nuclei of HL-60 cells treated with MNase after permeabilization with lysolecithin. The scale bar represents 5 µm. The percentage of nuclei exhibiting chromatin condensation is indicated below each panel (also for Figs 2-5). Except for Fig. 4, standard deviations were below ±5% of the mean values and are not shown.

FIG. 2. Energy and ionic strength requirements for chromatin condensation. DNA fragmentation (panel A) and chromatin condensation (panel B) of nuclei isolated from normal HeLa cells treated with activated DFF or MNase in the presence or absence of Zn$^{2+}$, ATP, ATPγS or pretreated with apyrase. DNA fragmentation (panel C) and chromatin condensation (panel D) in nuclei treated with activated DFF or MNase in the presence of either 100 or 10 mM KCl as indicated. To remove Mg$^{2+}$, nuclease-treated nuclei (samples 1 and 3, panels C and D) were incubated for 15 min on ice with 10 mM EDTA before fixing with formaldehyde. The scale bar represents 5 µm.
FIG. 3. Nuclease-induced chromatin condensation is inhibited by stabilizing nuclear components. DNA fragmentation (panel A) and chromatin condensation (panel B) in nuclei isolated from normal HeLa cells treated with MNase after reversible exposure to either CaCl₂, CuCl₂, diamide or pH 4. The scale bar represents 5 µm. Panel C – PAGE-SDS analysis of proteins extracted from nuclei after reversible exposure to stabilizing agents; Lane labels: T – total nuclear proteins; 1 – 0.2M NaCl extracts; 2 – 0.35M NaCl extracts. The percentage of extracted proteins is indicated below the stained gel.

FIG. 4. Partial inhibition of chromatin condensation by blocking F-actin depolymerization. DNA fragmentation (panel A) and chromatin condensation (panel B) in isolated nuclei incubated with 10 µM phalloidin and/or latrunculin A, and then digested with MNase. Nuclei treated with phalloidin and latrunculin A, or reversibly exposed to low pH, CaCl₂, CuCl₂ or diamide were digested with MNase, and then pelleted by low speed centrifugation. Pelleted nuclei (P) and corresponding supernatants (S) were analyzed by either SDS-PAGE (panel C) or Western-blotted with anti-actin antibody (panel D). DNA fragmentation (panel E) and chromatin condensation (panel F) in HL-60 cells treated with etoposide in the presence of 0.3 µM jasplakinolide and latrunculin A. Panel G – cells were labeled with BrdU prior to etoposide/jasplakinolide treatment, and then nuclei were stained with both DAPI and anti-BrdU antibody (secondary antibody conjugated with Texas Red). The scale bars represent 5 µm (panel B) or 20µm (panel F and G). Numbers below subpanels are the mean values ± S.D for 3-5 independent experiments.

FIG. 5. Effect of DNA groove-specific binding or intercalating agents on chromatin condensation. DNA fragmentation (panel A) and chromatin condensation (panel B) in nuclei
isolated from normal HeLa cells treated with MNase in the presence of 80 µM distamycin A, chromomycin A₃, bisbenzimide (Hoechst 33258), methyl green, ethidium bromide or etoposide. Alternatively, drugs were added for 15 min after incubation with the nuclease (post-incubation). The scale bar represents 5 μm. (Panel C) Inhibition of chromatin condensation in MNase-digested nuclei co-treated with different concentrations of DNA-binding drugs. DNA from nuclei incubated with 50 µM ethidium bromide, chromomycin A₃, distamycin A or 100 µM methyl green and digested with MNase (panel D) or DNase I (panel E) was analyzed on native 6% acrylamide or denaturing 7M urea/7.5% acrylamide (10:1 acrylamide/bis-acrylamide ratio) gels, respectively.
### A. Enzyme Digestion

<table>
<thead>
<tr>
<th>Nuclease</th>
<th>DFF</th>
<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ATPγS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Apyrase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

#### Untreated

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DFF</th>
<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
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#### +Zn/ATP

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>+Zn/ATP</td>
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<td>98%</td>
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#### +ATP

<table>
<thead>
<tr>
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<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
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<td>92%</td>
<td>99%</td>
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#### -ATP

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<thead>
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<tbody>
<tr>
<td>-ATP</td>
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#### +ATPγS

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<tbody>
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<td>+ATPγS</td>
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#### Apyrase

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<tbody>
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### B. Enzyme Digestion

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</tr>
</thead>
<tbody>
<tr>
<td>KCl [mM]</td>
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<td>10</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>3</td>
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#### 100 mM KCl

<table>
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<tr>
<th>Treatment</th>
<th>DFF</th>
<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>98%</td>
<td>99%</td>
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#### 10 mM KCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DFF</th>
<th>MNase</th>
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</thead>
<tbody>
<tr>
<td>10</td>
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<td>82%</td>
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### C. Enzyme Digestion

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</thead>
<tbody>
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<td>KCl [mM]</td>
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</tr>
<tr>
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<td>1</td>
<td>3</td>
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#### 100 mM KCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DFF</th>
<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>98%</td>
<td>99%</td>
</tr>
</tbody>
</table>

#### 10 mM KCl

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DFF</th>
<th>MNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>75%</td>
<td>82%</td>
</tr>
</tbody>
</table>

### D. Enzyme Digestion

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>MNase</th>
</tr>
</thead>
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<td>31%</td>
<td></td>
</tr>
<tr>
<td>3 + EDTA</td>
<td>52%</td>
<td></td>
</tr>
</tbody>
</table>
A. 

B. no pre-treatment

C. 

percentage of total nuclear proteins in extracts
Modeling apoptotic chromatin condensation in normal cell nuclei: Requirement for intranuclear mobility and actin involvement
Piotr Widlak, Olena Palyvoda, Slawomir Kumala and William T. Garrard

J. Biol. Chem. published online April 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201027200

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