

# Chromatin Condensation during Apoptosis Is Accompanied by Degradation of Lamin A + B, without Enhanced Activation of cdc2 Kinase

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**Abstract.** Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria which are used to identify apoptotic cells. However, comparable changes are also observed in interphase nuclei which have been treated with cell extracts from mitotic cells. In this respect it is known that in mitosis, the lamina structure is broken down as a result of lamin solubilization and it is possible that a similar process is happening in apoptotic cells. The experiments described in this study have used confluent cultures of an embryonic fibroblast cell line which can be induced to undergo either apoptosis at low serum conditions or mitosis.

Solubilization of lamin A+B was analyzed by immunoblotting and indirect immunofluorescence. These studies showed that in mitotic cells lamina breakdown is accompanied by lamin solubilization. In apoptotic cells, a small amount of lamin is solubilized before the onset of apoptosis, thereafter, chromatin condensation is accompanied by degradation of lamin A+B to a 46-kD fragment. Analysis of cellular lysates by probing

blots with anti-PSTAIR followed by anti-phosphotyrosine showed that in contrast to mitosis, dephosphorylation on tyrosine residues did not occur in apoptotic cells. At all timepoints after the onset of apoptosis there was no significant increase in the activation of p34<sup>cdc2</sup> as determined in the histone H1 kinase assay. Coinduction of apoptosis and mitosis after release of cells from aphidicolin block showed that apoptosis could be induced in parallel with S-phase.

The sudden breakdown of chromatin structure may be the result of detachment of the chromatin loops from their anchorage at the nuclear matrix, as bands of 50 kbp and corresponding multimers were detectable by field inversion gel electrophoresis (FIGE). In apoptotic cells all of the DNA was fragmented, but only 14% of the DNA was smaller than 50 kbp. DNA strand breaks were detected at the periphery of the condensed chromatin by *in situ* tailing (ISTAIL). Chromatin condensation during apoptosis appears to be due to a rapid proteolysis of nuclear matrix proteins which does not involve the p34<sup>cdc2</sup> kinase.

**A**POPTOSIS was originally defined as a program of morphological changes accompanying cell death during which the cells round up and the chromatin condenses, leading to the formation of crescent-shaped masses aggregating at the membrane. In parallel the nucleolus dissolves (Kerr, 1971). These initial changes are followed by cytoplasmic and nuclear compaction, and ultimately by fragmentation of the cell. The nuclear fragments formed in this process are still surrounded by the nuclear membranes (Kerr, 1971; Oberhammer et al., 1993a). As a common biochemical marker for apoptosis, an activation of a non-lysosomal, Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease has been suggested (Wyllie, 1980; Cohen and Duke, 1984). The response is so readily identifiable that endonuclease activation

was proposed to be the initiating event in the characteristic condensation of chromatin (Cohen and Duke, 1984). However, it has been shown that incubation of interphase nuclei in extracts prepared from a mitotic cell population induces condensation of the chromatin, dissolution of the nuclear membrane and lamin phosphorylation (Nakagawa et al., 1989; Wood and Earnshaw, 1990). All of these events are most probable due to the activation of p34<sup>cdc2</sup> kinase and consequent phosphorylation of various nuclear substrates, like the lamins (Gerace and Blobel, 1980; Peter et al., 1990) and histone H1 (Nurse, 1990).

From a morphological point of view, chromatin condensation occurring in an interphase nucleus upon incubation in a mitotic extract looks rather similar to chromatin condensation occurring in an apoptotic nucleus. Furthermore it has been reported that the lamin filament gets phosphorylated during physiological cell death mediated by cytotoxic T-lymphocytes (Ucker et al., 1992).

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We therefore decided to compare activation of p34<sup>cdc2</sup> kinase and lamin solubilization during apoptosis and mitosis. A prerequisite was to establish a cell culture system where cells were undergoing apoptosis in a synchronous manner. For this purpose we have used an embryonic fibroblast system. Due to the spindle shape of these cells the processes of rounding up and detachment occur rather slowly as the cells undergo apoptosis at low serum conditions. Thus, these cells stay on the monolayer during the initial process of chromatin condensation. This allows an easy observation of the breakdown of the nuclear lamina by immunohistochemistry and detection of DNA fragmentation by in situ tailing (ISTAIL)<sup>1</sup>. Furthermore, these cells can be forced to undergo mitosis after aphidicolin block and thus provides an ideal cell line for comparing apoptosis and mitosis.

## Materials and Methods

### Materials

Anti Lamin A+B and p9:agarose beads (p9-beads) were a generous gift from E. Nigg (ISREC, Lausanne, Switzerland), anti-PSTAIR a kind gift from M. Yamashita (National Institute for Basic Biology, Okazaki, Japan). Materials for ISTAIL were acquired from Boehringer Mannheim, FRG. Secondary antibodies for the ECL-detection system and reagents were obtained from Amersham, UK.

### Cell Culture and Synchronization Experiments

**Cell lines.** The cell line (H11-rasR3) was obtained from primary rat embryo cells transfected with a plasmid coding for human papilloma virus (HPV) type 11 and a plasmid coding for a mutated ras gene. These cells divide rapidly for the first few passages, and then revert to a slow growing phenotype with no HPV11 and ras expression being detectable by Northern blots (Cerni et al., 1990). For routine culture, cells were diluted 1:4 (doubling time 36 h) and grown in DMEM medium supplemented with 10% FCS and 200 µg/ml gentamicin (G-418 sulphate). In all experiments, epidermal growth factor (EGF, 10 ng/ml) was added.

### Cell Culture Experiments

For induction of apoptosis at confluence H11-rasR3 cells were diluted 1:5 and grown for 4 d to reach 80% confluency. These cells were refed with medium for an additional 40 h before induction of apoptosis at low serum conditions. These were achieved by washing the monolayer twice with unsupplemented DMEM before addition of DMEM supplemented with 0.25% FCS.

For synchronization before S-phase (Figs. 6–8), cells were grown for 4 d to reach 80% confluency. They were refed with medium supplemented with 2 µg/ml aphidicolin. After 14 h, cells were released from the aphidicolin-block with two washings with DMEM and directed either into S-G2-M by adding medium supplemented with 10% FCS or into apoptosis by medium supplemented with 0.25% FCS. To follow the onset of DNA synthesis 10 µM BrdU was added.

For preparation of a mitotic cell population (Fig. 3), monolayers were split 1:15 and grown for 3 d. They were blocked at the entry of S-phase as described above. After 14 h, cells were released and directed into S-G2-mitosis by adding medium supplemented with 0.5 µg/ml nocodazole to arrest cells in mitosis. The monolayers were harvested after an additional 16 h.

### Quantification of Apoptotic Nuclei

Glass slides of 10-mm diam were put into 24-well plates or 100-mm dishes before plating. At the indicated time points they were removed and cells

fixed with 3% paraformaldehyde and washed with distilled water before air drying. Samples were stained with 8 µg/ml H33258 for 5 min, washed with distilled water, and mounted in Mowiol. At each time point two samples were analyzed by counting the number of normal nuclei, apoptotic nuclei, and mitotic figures (M) in 10 microscopic fields (~1,500 total nuclei). Apoptotic nuclei were further characterized as either with condensed chromatin (AN) before or as nuclear fragments (F) after nuclear fragmentation.

### Electron Microscopy

Cells grown on glass slides were forced to undergo apoptosis. Detaching apoptotic cells were separated from cells in the monolayer and fixed with 2.5% sodium phosphate buffered glutaraldehyde, pH 7.4 and processed as described previously (Oberhammer et al., 1993a).

### Immunohistochemistry

For detection of lamins by indirect immunofluorescence (see Fig. 3), cells grown on glass slides were fixed with 3% paraformaldehyde, washed with distilled water, and air dried. Immunostaining with anti-lamin A+B, or anti-lamin A+C (1:300 dilution) was performed after permeabilization of cells with 0.5% Triton X-100 as described by Nakagawa et al. (1989), followed by incubation with a Texas red-linked anti-mouse Ig (1:500 dilution, Amersham, UK). Samples were counterstained with H33258 (8 µg/ml) before mounting in Mowiol.

For detection of incorporated BrdU (see Fig. 8), cells had to be fixed in 70% ethanol at -20°C. DNA was denatured for 10 min with 2N HCl at 37°C and the samples neutralized with several washings with 0.1 M HCl, 0.1 M NaBorate, pH 8.5. Anti-BrdU (Boehringer-Mannheim, FRG) was used at a concentration of 1:100, followed by Texas red-linked anti-mouse Ig. The samples were counterstained with H33258 (8 µg/ml) before mounting in Mowiol. For quantitative evaluation, the unlabeled antibody peroxidase-antiperoxidase technique (Sternberger et al., 1970) was used for detection and samples counterstained with hematoxylin.

### In Situ Tailing

ISTAIL (see Fig. 9) on isolated nuclei was carried out using a modified procedure of the method described by Gold et al. (1993). For rendering the target accessible, we pretreated the fixed samples with a chaotropic agent before digestion with proteinase K. Cells grown on glass slides were fixed 5 min with 3% paraformaldehyde, washed with distilled water, and dried at room temperature. Samples were stored at -20°C. Slides were pretreated with Target Unmasking Fluid (TUF-TM, Kreatech Biotechnology, Netherlands). Cells were deproteinized with 20 µg/ml proteinase K for 20 min and sequentially washed with PBS and TBS, pH 7.4. At this stage, control samples were treated with 1 µg/ml DNase I in Tris, 10 µg/ml BSA, pH 7.4 for 20 min.

Samples were incubated with digoxigenin-DNA labeling mixture and terminal deoxynucleotidyl transferase (0.5 U/µl) in TRIS, 10 µg/ml BSA, 1 mM cobalt chloride, pH 7.4 for 1 h at 37°C. The reaction was stopped with Tris, 20 mM EDTA, pH 7.4 and washed extensively with TBS. An anti-digoxigenin alkaline phosphatase labeled (Fab fragments) was used for detection of digoxigenin incorporated into DNA. The alkaline phosphatase developed with Fast Blue Salt until a deep blue stain was obtained (10 min) in positive controls. The cells were counterstained with HOECHST 33258 before mounting in Mowiol.

### Preparation of Samples for Agarose Gel Electrophoresis, Immunoblotting, and Precipitation of p34<sup>cdc2</sup> Kinase

Cells were plated onto 100-mm dishes containing two glass slides (10-mm diam) and forced to undergo apoptosis or mitosis. At the indicated time points, the glass slides were taken and the number of normal, apoptotic nuclei and mitotic figures quantified. Cells were scraped from the dish in 5 ml ice-cold PBS supplemented with aprotinin, leupeptin, PMSF (5 µg/ml), and 0.05 M NaF. In the time course experiments apoptotic cells from the supernatant were harvested by centrifugation and combined with cells in the monolayer. Apoptotic cells were allowed to detach into the supernatant after renewal of the serum free DMEM. After one hour these freshly formed apoptotic cells were collected by centrifugation (A). Cells were pelleted at 1,000 g and suspended in 1 ml TBS supplemented with aprotinin, leupeptin, PMSF (20 µg/ml), and 0.1 M NaF (ALPNaF).

1. **Abbreviations used in this paper:** AN, apoptotic nuclei; DSB, double strand breaks; F, fragment; FIGE, field-inversion gel electrophoresis; HPV, human papilloma virus; ISTAIL, in situ tailing; M, mitotic.

## Gel Electrophoresis

**Conventional agarose gel electrophoresis.** Cells ( $2.5 \times 10^6$ ) were collected by centrifugation and suspended in 100  $\mu$ l of L-buffer (0.01 M Tris-HCl, 0.1 M EDTA, 0.02 M NaCl, pH 7.4) at 37°C and 100  $\mu$ l of prewarmed 1% In Cert Agarose in L-buffer (FMC) was added. Four plugs were formed on ice for 10 min, and then transferred into 5 ml 1% sarcosylate, 20  $\mu$ g/ml proteinase K in L-buffer and digested overnight at 37°C. The plug and the supernatant were separated. DNA in the supernatant was precipitated with 0.5 vol 7.5 M ammonium acetate and 2 vol of absolute ethanol at -20°C. After centrifugation DNA was dissolved in 100  $\mu$ l of 10 mM Tris, 10 mM EDTA, pH 8.0. 10  $\mu$ l was used for conventional gel electrophoresis or determination of DNA content with H33258. After electrophoresis, the gel was stained with ethidium bromide. Gels were incubated with 0.5  $\mu$ g RNase-A/ml in running buffer for 2-3 h at 37°C.

## Field-inversion Gel Electrophoresis

Field-inversion gel electrophoresis (FIGE) was carried out under the following conditions. Gels were run at 200 V in 0.5\* TBE (89 mM Tris, 50 mM boric acid, 2 mM EDTA, pH 8.2) with a pulse rate from T1 equal to 2.4 s for the first 3 h and with a ramping rate from T1 equal to 2.4 s to T2 equal to 84 s for the next 24 h, always with a forward to backward ratio of 3 at 4°C using 1.4% pulsed field certified agarose (Biorad Labs., Hercules, CA). After electrophoresis, the gel was stained with ethidium bromide. Molecular weight markers (Lambda DNA ladder, *Saccharomyces cerevisiae* chromosomal DNA) were purchased from FMC.

## Precipitation of p34<sup>cdc2</sup> Kinase and In Vitro Histone H1 Kinase Assay

Cells ( $2.5 \times 10^6$ ) were collected by centrifugation and suspended in 1 ml of lysis buffer (50 mM TRIS, pH 7.4, 125 mM NaCl, 1 mM DTT, 0.2% NP-40, 5 mM EDTA, 5 mM EGTA, ALPNaF). After homogenization through a 1-ml syringe equipped with a 25 gauge needle, samples were left on ice for an additional 20 min. The cytosol was separated from the nuclear pellet by centrifugation at 1,000 g and used for the in vitro kinase assay.

Unspecific adsorption was prevented with the use of HiTrap Protein A columns (Pharmacia LKB Biotechnology, Piscataway, NJ) before incubation of 150  $\mu$ g cytosolic extract protein in 1 ml lysis buffer with 30  $\mu$ l of a 50% suspension of p9-Sepharose beads. After extensive washings with lysis buffer the beads were separated in two portions, one was used to measure histone H1 kinase activity, the other for immunodetection of p34<sup>cdc2</sup> with anti-PSTAIR (as control). The histone H1 kinase assay was performed essentially as described by Peter et al. (1990).

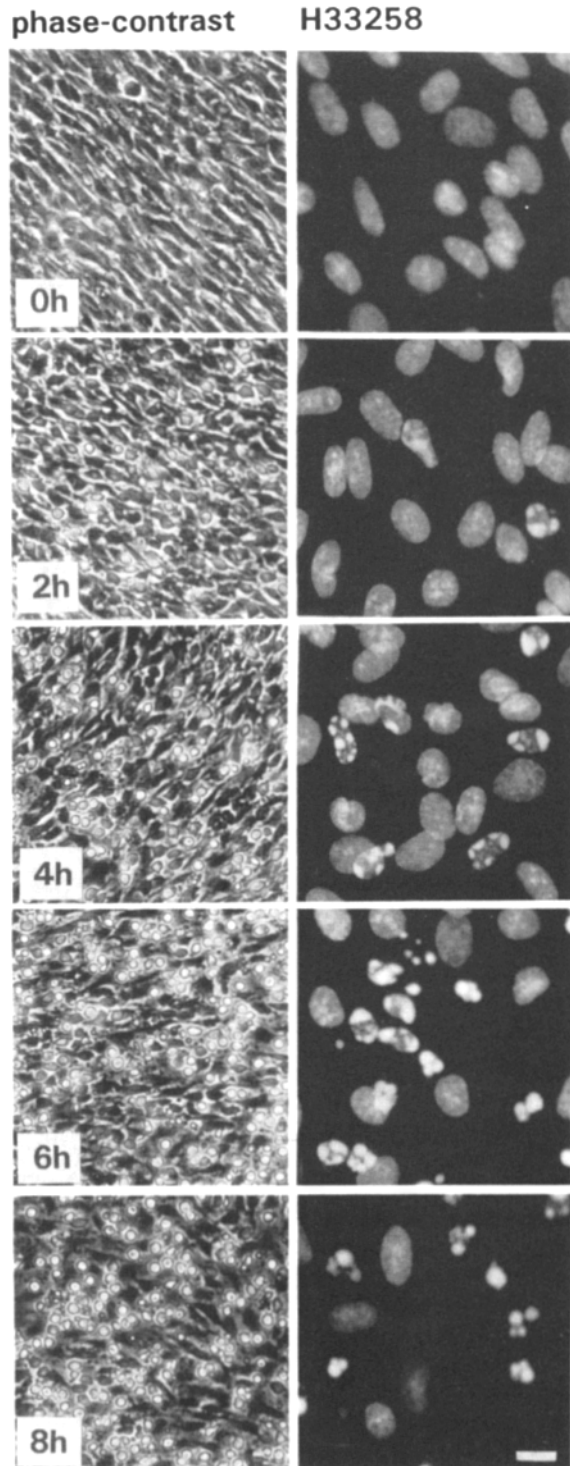
## Immunoblotting

The frozen cells were suspended in 200  $\mu$ l RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 2 mM MgCl<sub>2</sub>, ALPNaF), and 10  $\mu$ l taken for protein determination. One portion of 100  $\mu$ l was treated with 300  $\mu$ l sample buffer according to Laemmli (1970) before boiling at 95°C for 10 min (i.e., lysate, L). To the remaining 100  $\mu$ l, Triton X-100 and 2.5 M NaCl were added to give a final concentration of 1% (vol/vol) and 500 mM, respectively. After 20 min on ice the samples were centrifuged and the pellet resuspended in 200  $\mu$ l RSB. Aliquots (10  $\mu$ l, corresponding to 8  $\mu$ g protein) of both pellet (P) and supernatant (S) were analyzed by SDS-PAGE and Western blotting. Membranes were either probed with anti-lamin A+B or first probed with anti-phosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY or Biomakor, Israel) followed by reprobing with anti-PSTAIR after removal of the first antibody detection system with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7.

The following conditions were used for antibody binding: anti-phosphotyrosine, 1:10,000 dilution, 4°C, 16 h; anti-lamin A+B, 1:3,000 dilution, 1-2 h; anti-PSTAIR, 1:5,000 dilution, 1-2 h; anti-mouse peroxidase linked (Amersham Corp., Arlington Heights, IL), 1:4,000 dilution, 1 h. The bound secondary antibody was detected using the enhanced chemiluminescence Western blotting detection system (Amersham, UK). All steps including probing with antibodies were performed according to the manufacturer's instructions. Films were scanned using laser densitometry.

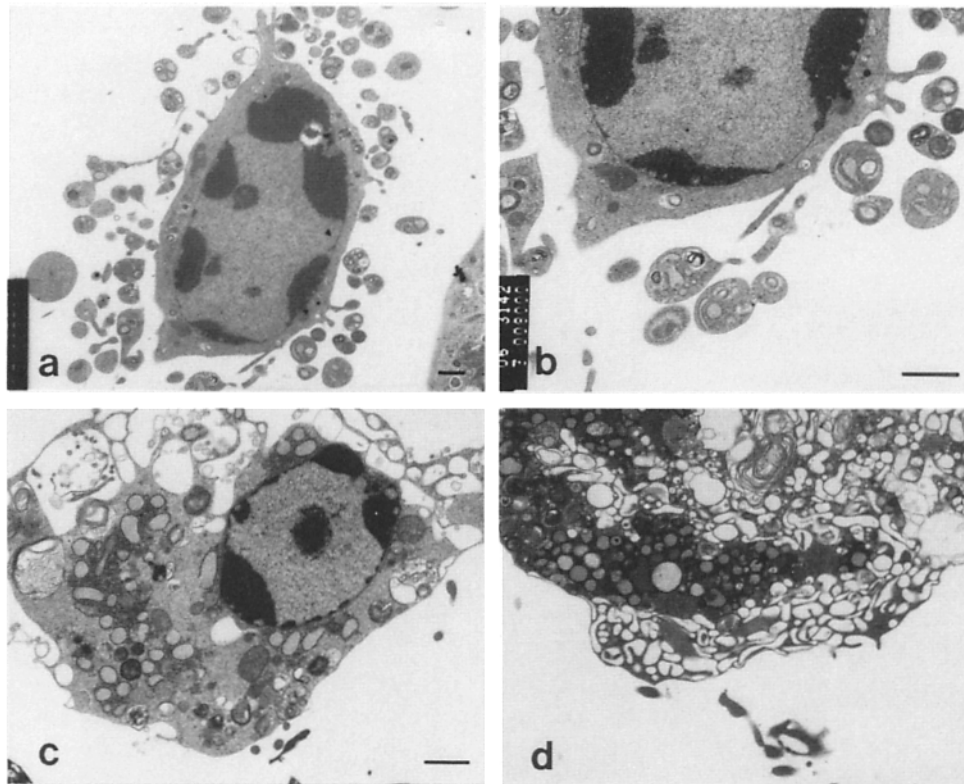
## Miscellaneous

Protein was determined with the Biorad Assay. DNA content was determined by quantification of the fluorescent H33258-DNA complex in 10 mM



**Figure 1.** Morphology of confluent monolayers at low serum conditions. Confluent monolayers at low serum conditions were prepared as described in Materials and Methods and the cells observed by phase-contrast microscopy (*phase contrast*) before staining the DNA with H33258 (*H33258*). For a better demonstration of the chromatin condensation in apoptotic cells, the H33258 stained nuclei are shown at a higher magnification. Bars, 10  $\mu$ m.

Tris, 10 mM EDTA, 0.2% SDS, pH 8.0 (10 min, 1,000 rpm) using calf thymus DNA for standardization (Labarca and Paigen, 1980).



**Figure 2.** Electron microscopy of newly formed apoptotic cells. Confluent monolayers at low serum conditions were prepared as described for Fig. 1 and examined by electron microscopy. Apoptotic cells in the monolayer (*a* and *b*) or after detachment (*c* and *d*). Higher magnifications show the intact nuclear envelope (*b*) or intracellular vacuoles (*d*). Bars, 1  $\mu\text{m}$ .

## Results

### *Establishment of a Synchronous System of Cells Undergoing Apoptosis Using an Embryonic Fibroblast upon Transfection with v-ras Together with HPV11*

For the purpose of this study it was necessary to have a cell line which undergoes apoptosis in a synchronous manner. In preliminary studies we screened a number of epithelial and fibroblast cell lines for their sensitivity to low serum conditions. These experiments showed that cultures of the H11-rasR3 cell line executed an immediate response to low serum conditions which seemed to be more noticeable in areas of high cell density. We therefore prepared confluent monolayers by growing cells until nearly reaching confluence, and then refeeding for an additional 40 h (approximately the duration of one cell cycle) in the presence of EGF. Apoptosis was then induced by low serum conditions and the incidence of cell death determined at different times by phase contrast microscopy and after staining of the same cultures with H33258 (Fig. 1).

Within one hour at low serum conditions, many of the cells showed a change in shape from a flat spindle-like appearance to a rounded one leaving intercellular spaces in the monolayer. This was followed by a more pronounced rounding up (4–6 h), leading finally to detachment of the cells (8–10 h). The detached cells were small with condensed chromatin as shown by phase contrast microscopy and H33258 staining. The morphology of these cells is characteristic of apoptosis.

Electron microscopy (Fig. 2) showed that the nuclear envelope during the whole process of chromatin condensation remained intact. This was true also for the detached

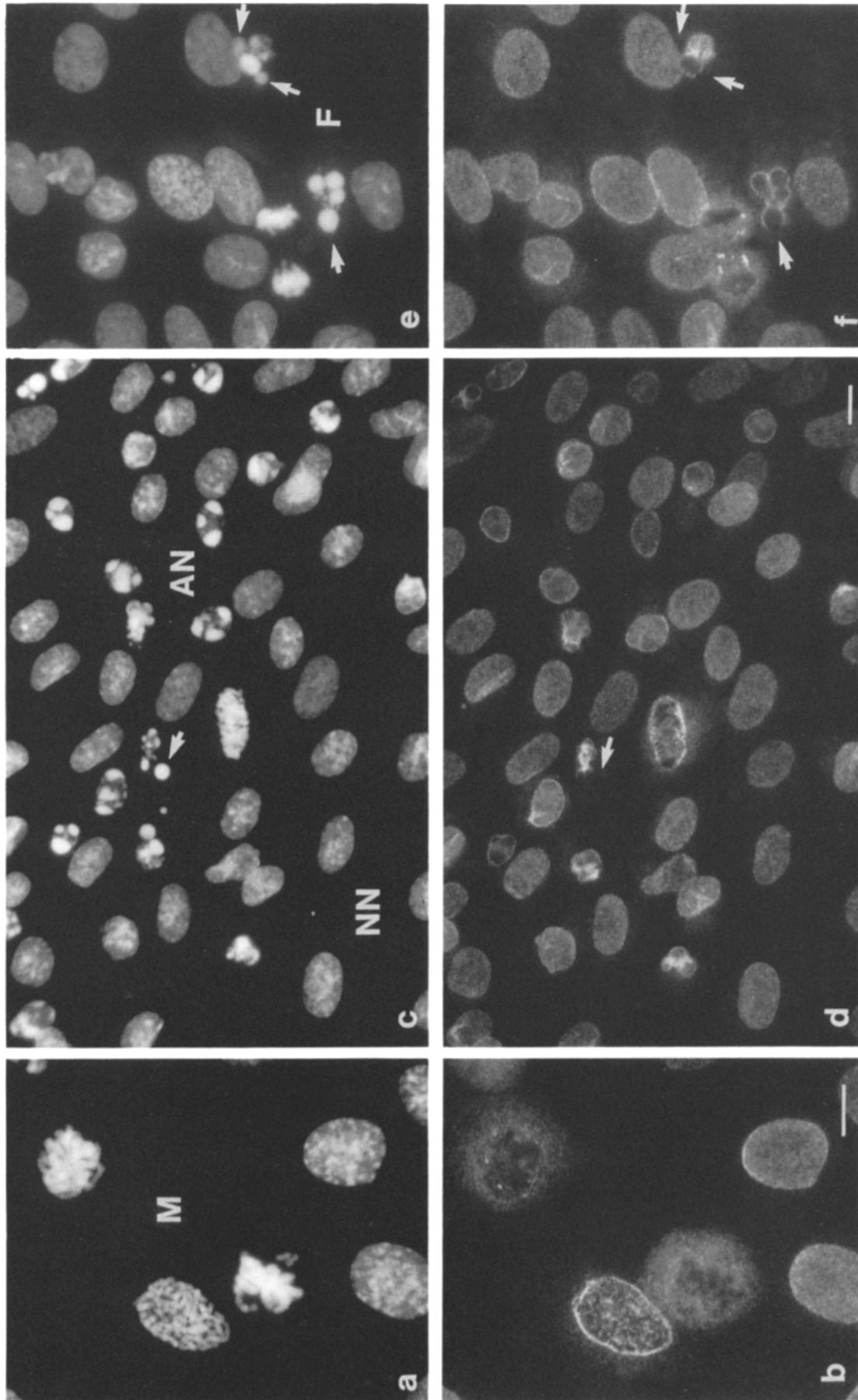
apoptotic cells which had started to develop intracellular blebs.

### *Comparison of Lamin Solubilization and Activation of P34<sup>cdc2</sup> Kinase in Apoptotic and Mitotic Cultures*

During mitosis the nuclear lamins are phosphorylated by the activated p34<sup>cdc2</sup> kinase. This leads to dissolution of the polymer structure by formation of lamin monomers (Gerace and Blobel, 1980; Peter et al., 1990). After dissolution of the nuclear membrane, the monomer dissolves into the cytoplasm and in case of lamin B it has been shown that it is associated with membrane vesicles (Gerace and Blobel, 1980; Stick et al., 1988). We have confirmed these observations in our cell line by indirect immunofluorescence of lamin A+B in mitotic cultures (M) prepared 8–10 h after release from aphidicolin block (Fig. 3). In prophase, the stage where the chromosomes have already condensed and the nuclear membrane has not dissolved yet, lamin A+B condensed in between the chromosomes and at the nuclear periphery. In later stages the epitope was diffusely detected in the cytoplasm.

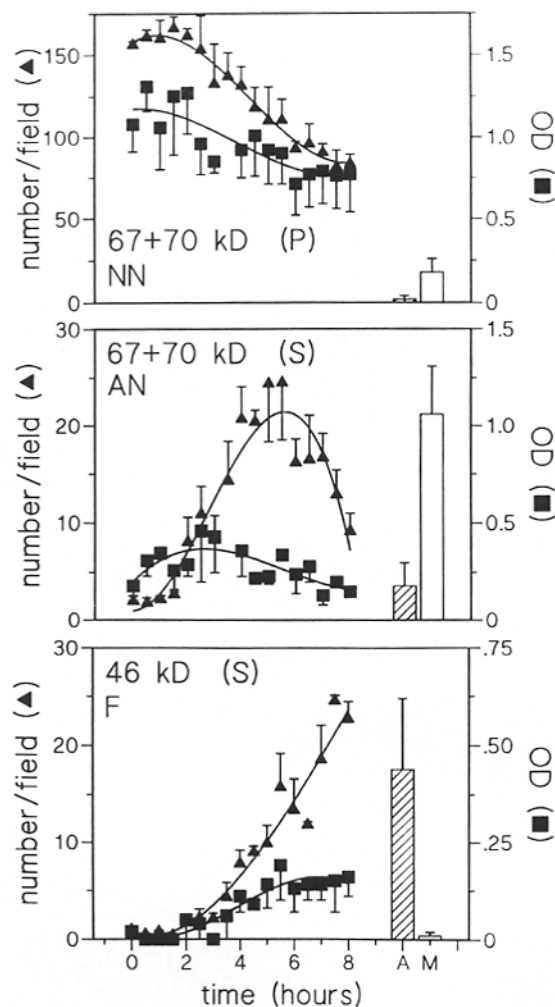
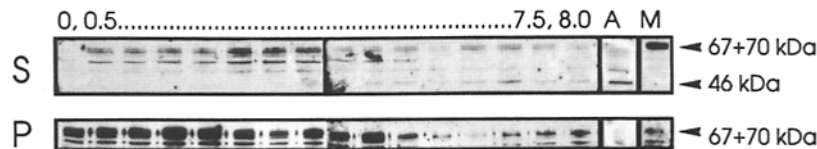
In apoptotic nuclei (AN), immunofluorescence of lamin A+B was localized around the condensed chromatin. After nuclear fragmentation (F) the epitope seemed to be less and sometimes undetectable. Similar results were observed using anti lamin A+C (data not shown).

Solubilization of lamin A+B was analyzed by centrifugation and separation into soluble (S) and pelletable (P) fractions. The pelletable fraction represents the intact or polymerized lamin and is identified as the 67+70 kD bands on SDS gel electrophoresis (Fig. 4). The soluble fraction contains two identifiable lamin products; the depolymerized



**Figure 3.** Indirect immunofluorescence of lamin A+B in mitotic and apoptotic cells. Mitotic or apoptotic cell cultures were prepared and the lamin distribution examined by direct immunofluorescence as described in Materials and Methods. Cultures containing a high incidence of mitotic cells 10 h after release from aphidicolin block are shown in *a* and *b* and apoptotic cells 6 h at low serum conditions are shown in *c-f*. Chromatin condensation was visualized with H33258 dye staining (*a*, *c*, and *e*) and lamin A+B by Texas-red fluorescence (*b*, *d*, and *f*). Various nuclear structures are indicated as: *M*, mitotic figure; *NN*, normal nucleus; *AN*, apoptotic nucleus with condensed chromatin; *F*, fragmented apoptotic nucleus. Arrows indicate different stages of lamin degradation. Bars, 10  $\mu$ m.

lamin A+B



lamin monomers i.e., 67+70 kD and the degraded monomer fractions which have a molecular mass of 46 kD.

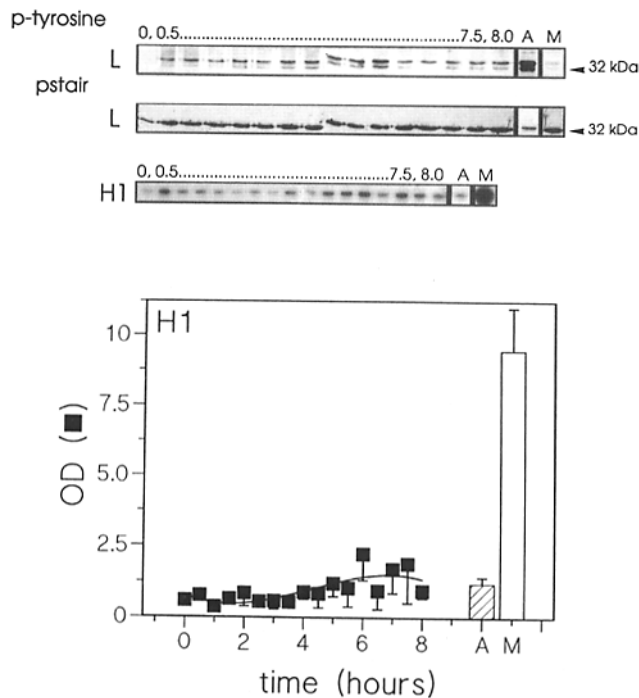
For the time course experiment the detaching cells were combined with cells harvested from the monolayer. For comparison, positive controls were prepared from detached apoptotic cells (*shaded histogram*) and mitotic cells from monolayers blocked in mitosis with nocodazole (*unshaded*). The latter cells were morphologically mitotic and the predominant lamin form was (as expected) the soluble 67+70 kD form ( $83 \pm 6\%$ ). The apoptotic cells contained predomi-

nantly fragmented nuclei with some condensed chromatin structures and in this case the lamins were mainly found as the degraded 46-kD form ( $78 \pm 10\%$ ).

In the time course experiment (Fig. 4) there is a distinct correlation with the morphological changes and the pattern of lamin degradation. Thus, as the number of normal nuclei decreases to  $51 \pm 4\%$ , there is a parallel decrease in the intact lamin structure to  $64 \pm 18\%$ . The increase in apoptotic nuclei is preceded by a small increase in soluble lamins (67+70 kD, maximum 30% at 4 h), and the production of

**Figure 4.** Analysis of solubilization of lamin A+B by Western blot and correlation with apoptosis. Cells were forced to undergo apoptosis at low serum conditions. At the indicated times, cells were harvested from both the monolayer and media (i.e., detached cells). Cell lysates were prepared by Triton X-100 + 0.5 M NaCl lysis of the cells and analyzed by SDS gel electrophoresis and Western blotting as described in Materials and Methods. Endpoint controls were prepared by harvesting only the detached apoptotic (A) cells in the culture media and those cells arrested in mitosis (M) after nocodazole inhibition. Solubilization of lamin A+B was determined by immunoprobings with lamin A+B (*lamin A+B*) of the soluble (S) and pelletable (P) fraction obtained after centrifugation of the cell lysate. Quantitation of the lamin sub-units was determined by laser densitometric scanning of the films exposed to the blot after application of the ECL-detection system. These sub-units are shown as (67+70 kD) in the pelletable (P) and soluble fraction (S) and as the 46-kD fragment in the soluble (S) fraction. Each culture dish also contained  $2 \times 10$  mm glass cover slides which were used for morphological analysis after staining with H33258. The number of normal nuclei (NN); apoptotic nuclei with condensed chromatin (AN); and fragmented apoptotic nuclei (F) were counted. Densitometric analysis of apoptotic (A) or mitotic (M) samples is shown as histograms. NN, AN, or F (▲); 67+70 or 46 kD (■). Means  $\pm$  SEM of four experiments are shown.





**Figure 5.** Analysis of p34<sup>cdc2</sup> activation. Cells were forced to undergo apoptosis by low serum conditions and harvested as described in Fig. 4 at the indicated times. For comparison, apoptotic cells detaching into the media (A) and cells arrested at mitosis (M) were analyzed. Tyrosinylation of p34<sup>cdc2</sup> kinase was detected in cellular lysates (L) by immunoprobings with anti-p-tyrosine (p-tyrosine) followed by reprobings with anti-pstairs (pstairs). For the histone H1 kinase assay (H1), p34<sup>cdc2</sup> was precipitated with p9-beads and the precipitated complex used as described in Materials and Methods. Quantitation of phosphorylation of p34<sup>cdc2</sup> on tyrosine and histone H1 activity was carried out by laser densitometric scanning (H1) of the exposed films. Densitometric analysis of apoptotic (A) or mitotic (M) samples is shown as histograms. Means  $\pm$  SEM of four experiments are shown.

fragmented nuclei is accompanied by degradation of the lamins to the 46-kD fragment. These results demonstrate that the morphological changes in which the nuclei progress from the normal state to condensed chromatin to fragmented nuclei is paralleled by the progressive degradation of the lamin network.

Activation of p34<sup>cdc2</sup> kinase before mitosis occurs by a specific dephosphorylation on tyrosine-15 and threonine-14 after association with cyclin B (Nurse, 1990). We investigated the dephosphorylation on tyrosine residues by Western blots. First the membranes were probed with anti-phosphotyrosine, followed by reprobings with anti-PSTAIR (Fig. 5). Anti-PSTAIR detects the most conserved amino acid sequence of p34<sup>cdc2</sup>. With this method no dephosphorylation of the p34<sup>cdc2</sup> kinase was evident after low serum conditions. After normalization of the scan of the 32-kD phospho-tyrosine band to its p34<sup>cdc2</sup> counterpart even a slight increase in the phosphorylation of the kinase was detected in apoptotic cells (0 h,  $1.0 \pm 0.6$ ; M,  $0.6 \pm 0.2$ ; A,  $3.2 \pm 0.9$ ).

The active p34<sup>cdc2</sup> kinase associates not only with the cyclins but also with p13suc or its vertebrate homologue p9

(Nurse, 1990). We used this protein for precipitation of p34<sup>cdc2</sup> and analyzed its histone H1 kinase activity. The kinase showed a slight increase in activity in parallel but not before the increase of apoptosis. However, in apoptotic cells histone H1 kinase activity ( $1.2 \pm 0.22$ ) was  $\sim$ 10-fold lower than that of mitotic cells ( $9.46 \pm 1.55$ ).

### Induction of Apoptosis after Release of Cells from Block at S-phase Entry by Aphidicolin

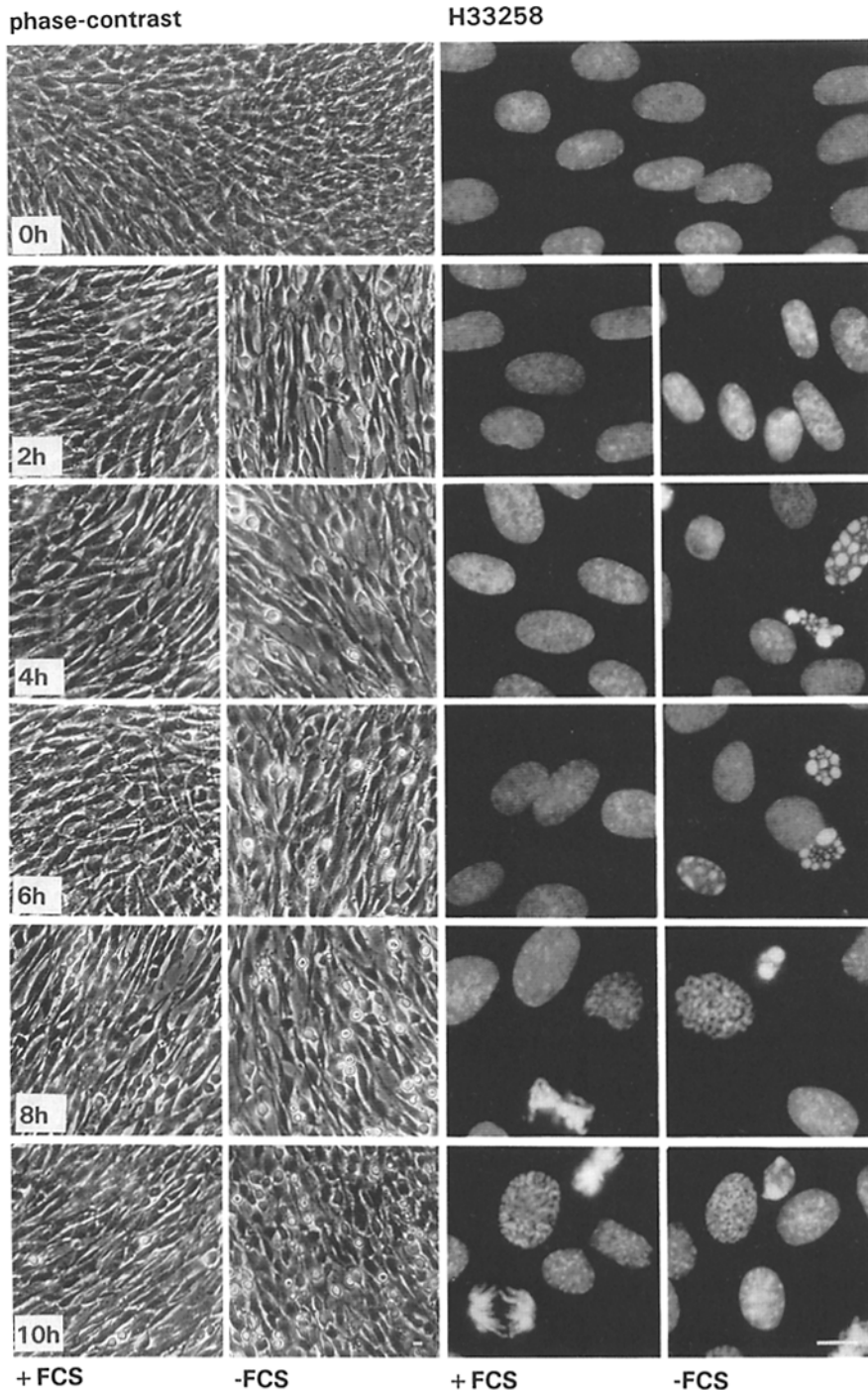
The results shown above suggest that activation of p34<sup>cdc2</sup> kinase apparently does not lead to the chromatin condensation during apoptosis. We furthermore wanted to know if apoptosis and mitosis can be induced in parallel at low serum conditions after release of cells from aphidicolin-block. For comparison, cells were further treated with serum. The incidence of cell death and mitosis was determined by phase contrast microscopy and after staining of the same cultures with H33258 (Figs. 6 and 7). In the first hours at low serum conditions apoptotic cells had a classical appearance of chromatin condensation, whereas later on nuclei with various patterns of chromatin condensation could be observed (Fig. 6). At 4 h at low serum conditions the incidence of apoptotic nuclei rose to  $12.8 \pm 1.7\%$  (Fig. 7) and stayed at this percentage thereafter ( $14.7 \pm 0.3\%$  10 h at low serum conditions). Both in serum-treated and serum-deprived cultures, comparable numbers of cells started to undergo mitosis 6 h after release from the aphidicolin block (Figs. 6 and 7). This led to the paradox that 10 h after release of the S-phase block the rate of mitosis was slightly higher in the apoptotic cultures ( $3.7 \pm 0.9$ ) than in the mitotic ones ( $3.0 \pm 1.3\%$ ).

For detection of cells in S-phase we looked at the incorporation of BrdU after release from aphidicolin-block with an immunohistochemical technique (Fig. 8). We noticed that at low serum conditions cells were able to replicate DNA. Apoptotic cells positive for BrdU could be observed. This indicates that these cells synthesized DNA before they received the signal to undergo apoptosis.

### Analysis of DNA Fragmentation

We wanted to compare the appearance of DNA double strand breaks (DSB) and further digestion of chromatin with the stages of chromatin condensation. We analyzed DNA strand breaks by ISTALL (Fig. 9) and pulse field gel electrophoresis followed by conventional gel electrophoresis (Fig. 10). In preliminary experiments we found that both the cellular and chromatin condensation prevented the access of enzymes and antibodies to their targets (Fig. 9 a). However, pretreatment of cells with a chaotropic agent followed by removal of proteins by proteinase K digestion rendered the condensed chromatin accessible to ISTALL and antibody labeling (Fig. 9 b). With this method no background digoxigenin labeling was detectable in normal nuclei. DSB were detectable during the initial stages of chromatin condensation. The digestion starts around the areas of condensed chromatin (Fig. 9 c). The complete genomic digestion takes hours and apoptotic cells detaching into the supernatant contain partly digested DNA (data not shown).

Fig. 10 shows the appearance of DNA fragments detected by gel electrophoresis. As expected from our experiments (Oberhammer et al., 1993b) the fragmentation of DNA into domain-sized fragments parallels the morphological onset of



**Figure 6.** Morphology of cells directed into mitosis or apoptosis after release from S-phase block. After release from the aphidicolin-block (0h) cells were either directed into S-G2-M by adding medium supplemented with 10% FCS (+FCS) or into apoptosis by medium supplemented with 0.25% FCS only (-FCS). The cells were observed by phase-contrast microscopy (*phase contrast*) before staining the DNA with H33258 (*H33258*). For a better demonstration of the chromatin condensation, H33258 stained nuclei are shown at a higher magnification. Bars, 10  $\mu$ m.

apoptosis (Fig. 10). The 50-kbp DNA is progressively degraded to smaller sizes as revealed by conventional gel electrophoresis of the short chain DNA migrating out of the plug during digestion with proteinase K. Quantification of this DNA did not show a linear correlation with the amount of apoptotic cells in the culture. In detached apoptotic cells most of the DNA consists of 300 and 50 kbp (Fig. 10) and only  $14 \pm 2\%$  of the DNA is  $\leq 50$  kbp.

### Discussion

It is well established that most of the mitotic processes are triggered by tyrosine dephosphorylation of the p34<sup>cdc2</sup> com-

plex leading to an increase in kinase activity enabling the complex to phosphorylate various substrates, like e.g., lamins or histone H1 (for reviews see Draetta, 1990; Nurse, 1990; Murray and Kirschner, 1989). Furthermore it has been shown that extracts containing activated p34<sup>cdc2</sup> kinase prepared from *Xenopus* eggs (Newport and Spann, 1987; Adachi et al., 1991), clam oocytes (Dessev et al., 1991) or cells synchronized in mitosis (Nakagawa et al., 1989; Wood and Earnshaw, 1990) activate the processes of chromatin condensation and lamin solubilization in isolated nuclei. Therefore we hypothesized that some of the processes responsible for mitotic chromatin condensation might also be activated during apoptosis. For this study, we established



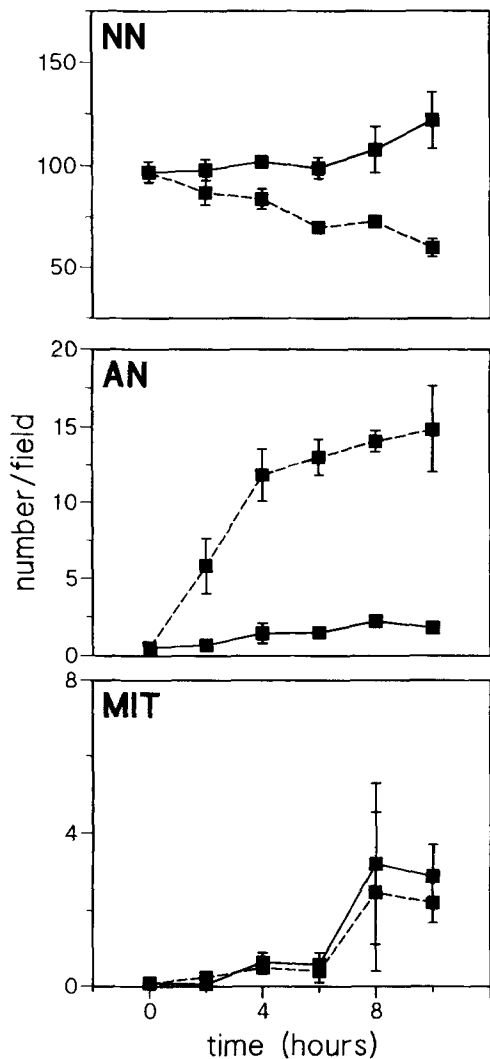


Figure 7. Quantification of mitosis and apoptosis in cultures directed into mitosis or apoptosis after release from S-phase block. After release of cells from the aphidicolin block and further treatment with serum (—) or without serum (---), normal nuclei (NN), apoptotic nuclei (AN+F), and mitotic figures (M) were

a highly synchronized system of H11-rasR3 cells undergoing apoptosis and examined the changes in lamin structure. The results can be summarized as follows: (1) There is a small increase in lamin solubilization before the onset of apoptotic chromatin condensation. (2) Lamin degradation parallels chromatin condensation. (3) In apoptotic cells the tyrosine residues of p34<sup>cdc2</sup> are not dephosphorylated. (4) In apoptotic cells p34<sup>cdc2</sup> kinase is nearly 10-fold less active than in mitotic cells. (5) Apoptosis is more easily induced in cells in S-phase than in M-phase. (6) DNA strand breaks, assayed by ISTAIL, were detected in initial stages of chromatin condensation around the areas of condensed chromatin. (7) The appearance of high molecular weight DNA of multimers of 50-kbp bands, as detected by FIGE, parallels chromatin condensation. (8) Apoptotic cells contain mainly digested lamin A+B with the DNA degraded mainly into large fragments (300–350 kbp) with only a small amount  $\leq 50$  kbp (14%). From these findings we can make the following conclusions and these are discussed below.

#### Chromatin Condensation during Apoptosis Occurs without a Parallel Increase in p34<sup>cdc2</sup> Kinase

The data shown in this paper show that p34<sup>cdc2</sup> is only slightly activated during apoptosis and that apoptosis could be far more induced in parallel with S-phase than in parallel with M-phase. It has been shown that chromatin condensation and fragmentation of DNA into oligosomes characteristic of apoptosis can be induced in isolated nuclei by incubation in an extract prepared from cells after a sequential S-phase/M-phase synchronization (Lazebnik et al., 1993). Upon depletion of activated p34<sup>cdc2</sup> kinase by p9 the extract was still able to produce chromatin condensation and fragmentation of DNA. Furthermore, chromatin condensation and DNA fragmentation could not be observed if the extract was prepared from M-phase cells not presynchronized in S-phase with aphidicolin. This correlates well with our observations.

evaluated in H33258 stained cultures. Means  $\pm$  SEM of four experiments are shown.

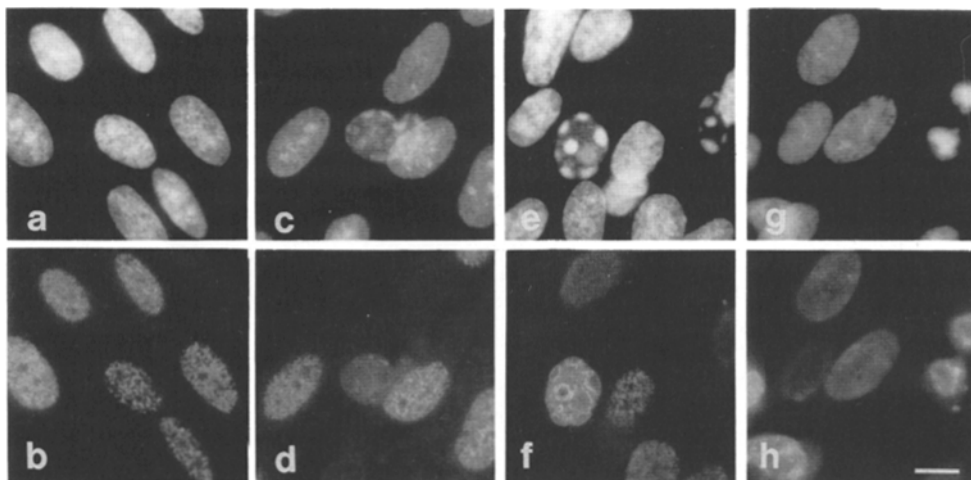
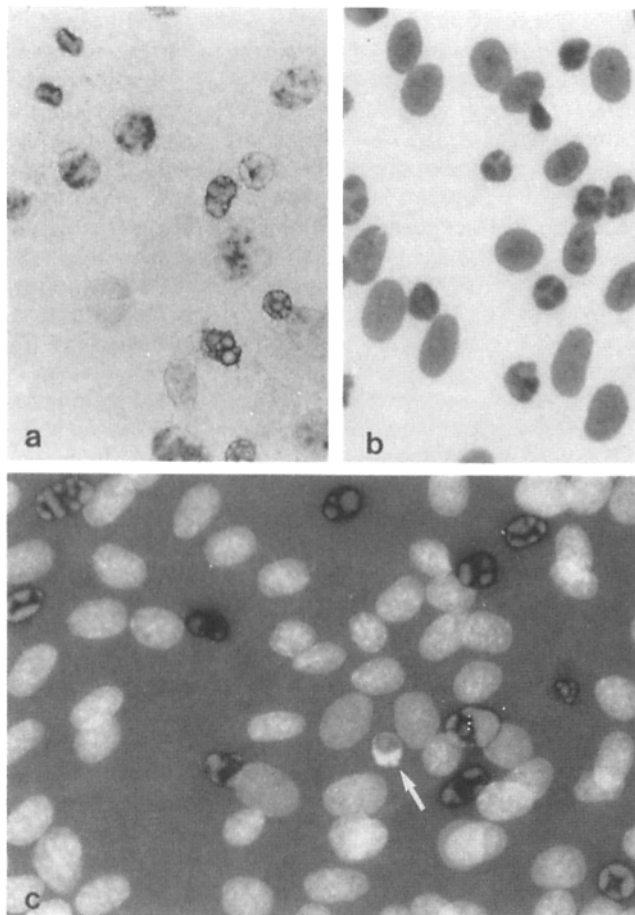


Figure 8. Incorporation of BrdU in cells directed into mitosis or apoptosis after release from S-phase block. After 14 h, cells were released from the aphidicolin-block with two washings with DMEM and directed either into S-G2-M by adding medium supplemented with 10% FCS (a and b) or into apoptosis by medium supplemented with 0.25% FCS (c-h). To follow the onset of DNA synthesis 10  $\mu$ M BrdU was added. At different time-points, cells were fixed with 70% ethanol and further processed for indirect immunofluorescence of BrdU. Stain-

ing with H33258 (a, c, e, and g) and with corresponding Texas-red fluorescence indicating brdU (b, d, f, and h) is shown for the timepoints which are 2 (a-d), 6 (e and f), or 10 h (g and h) after treatment. Bar, 10  $\mu$ m.

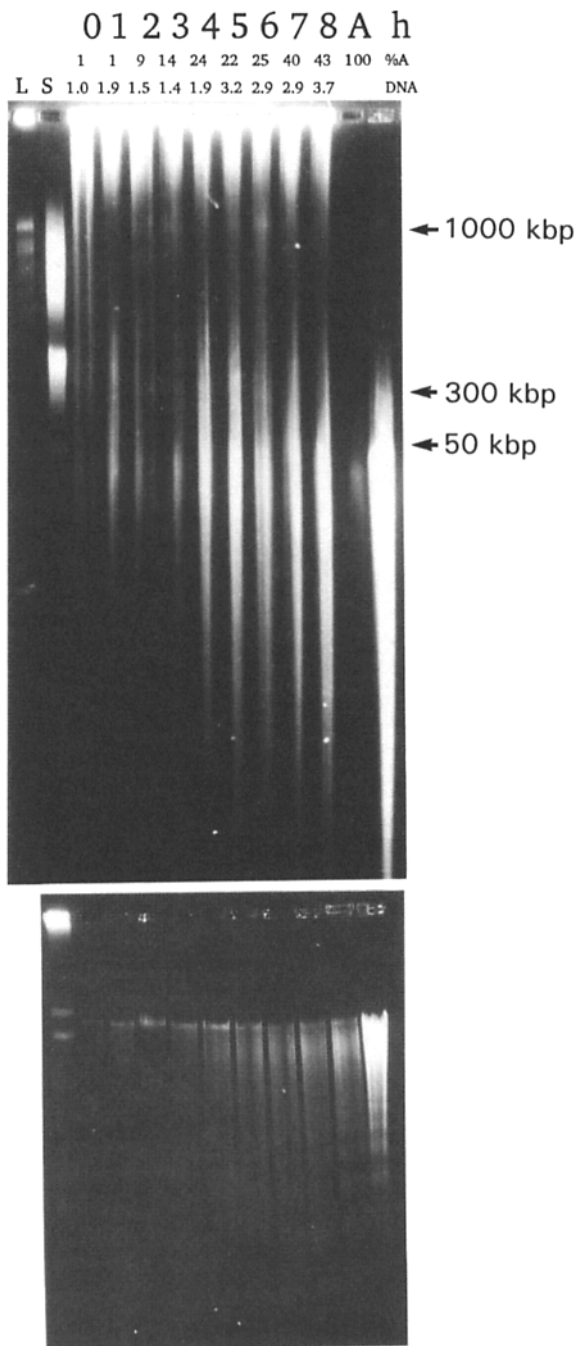


**Figure 9.** Detection of DNA DSB in apoptotic cells by ISTAIL. Apoptotic cells were prepared and ISTAIL carried out as described in Materials and Methods. The photomicrograph shows apoptotic cells visualized under normal and fluorescent light showing both the H33258 fluorescence and the Fast Blue stain indicating DSB. All samples were fixed with 3% paraformaldehyde 5 h after low serum conditions. Positive controls (*a* and *b*) were normal nuclei treated with DNase I. In *a* the pretreatment with the chaotropic agent which leads to swelling of the chromatin was omitted. The arrow in *c* indicates first stage of chromatin condensation in an apoptotic nucleus. Bar, 10  $\mu$ m.

### **Nuclear Lamins Are Degraded in Parallel with Chromatin Condensation**

Using anti lamin A+B we observed that the filamentous structure of the lamin network disappears during apoptosis, due to a time-dependent degradation. Digestion of nuclear lamin has also been found in physiological cell death mediated by cytotoxic T-lymphocytes (Ucker et al., 1992) and apoptosis induced by etoposide (Kaufmann, 1989).

The identity of the enzyme(s) responsible for lamin degradation is unknown. However in the nuclear scaffold a  $Ca^{2+}$  dependent protease exists which shows a marked selectivity for lamins (Tökés and Clawson, 1989). The localization and activity of lamin degradation ( $\sim 70\%$  of the lamins are destroyed within 60 min) of this enzyme would make it a good candidate for digestion of nuclear proteins during apoptosis.



**Figure 10.** Conventional agarose and FIGE in apoptotic nuclei. Apoptotic cells were prepared as described in Fig. 4. Before analysis at the indicated times, cells were harvested from both the monolayer and the media. As in Fig. 4, for comparative purposes, apoptotic cells (*A*) detaching into the supernatant were analyzed. The percentage of apoptotic cells (%*A*) indicates the percentage of apoptotic nuclei quantified in cells grown on glass 10-mm glass cover slides which were included in the normal culture dishes. Cells were embedded in agarose plugs and after digestion with proteinase K plugs were used for FIGE (*upper picture*). Short chain DNA having migrated out of the plug were quantified by the fluorescent H33258-DNA complex (*DNA*) analyzed by conventional gel electrophoresis (*lower picture*). As markers the Lambda DNA ladder (*L*) and *Saccharomyces cerevisiae* DNA standards (*S*) were used.

## Is DNA Fragmentation also a Result of the Proteolysis of Nuclear Proteins?

In the case of apoptosis mediated by etoposide it has been shown that there is a cleavage of the poly(ADP-ribose) Polymerase (Kaufmann et al., 1993) and digestion of other nuclear proteins like topoisomerase I and II, histone H1 and p67 (Kaufmann, 1989). Protease digestion of topoisomerase II removes its COOH-terminal end revealing a core enzyme with full decatenation activity (Shiozaki and Yanagida, 1991). Thus proteolysis of topoisomerase II during apoptosis could activate the protein. Topoisomerase II (Adachi et al., 1989; Earnshaw et al., 1985; Filipinski et al., 1990; Gasser and Laemmli, 1987) could be responsible for the formation of the large domain-sized (50 kbp) fragments of the DNA during apoptosis. In all cell lines studied so far, the appearance of these high molecular mass DNA bands detectable by FIGE (Walker et al., 1991; Brown et al., 1993; Oberhammer et al., 1993b; Tomei et al., 1993) parallels chromatin condensation, typical of apoptosis. It has been shown that incubation of interphase nuclei in mitotic extracts leads to different condensation patterns depending on the amount of Topoisomerase II present in a nucleus (Wood and Earnshaw, 1990; Adachi et al., 1991). In the *Xenopus* reconstitutive system, a nearly linear correlation between the degree of chromatin condensation and the amount of topoisomerase II was observed. Topoisomerases themselves are able to nick DNA around their specific DNA attachment sites (SAR regions) in the flanking regions (circe effect, Mirkovitch et al., 1984; Adachi et al., 1989). It has to be emphasized that in an apoptotic nucleus the amount of short chain DNA ( $\leq 50$  kbp) represents only a small amount of the total DNA. Thus a transient activation of topoisomerase II before its complete degradation could also account for the appearance of oligosomal fragments detectable by conventional gel electrophoresis.

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