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PROSPECTS

# Cells En Route to Apoptosis Are Characterized by the Upregulation of *c-fos, c-myc, c-jun, cdc2,* and RB Phosphorylation, Resembling Events of Early Cell-Cycle Traverse

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**Abstract** Density-arrested quiescent murine Balb/c-3T3 cells are dependent upon growth factors for their survival. Withdrawal of serum from their medium induces rapid cell death, the mechanism of which is not yet fully understood. We have studied the effect of serum deprivation on density-inhibited quiescent Swiss 3T3 cells and found that they undergo rapid cell death upon total withdrawal of serum. The nature of this cell death is similar to apoptosis, as shown by cellular and nuclear morphology and DNA fragmentation into oligonucleosomal fragments. Investigating the regulation of early cell-cycle genes during this process, we found that *c-myc, c-jun, c-fos,* and *cdc2* protein presence is induced after serum deprivation, when the phosphorylated form of the RB protein also appears. The upregulation of these genes' protein products is coupled with the appearance of PCNA, a proliferation-specific nuclear antigen, as well as significant incorporation of BrdU, which may reflect DNA repair activity; in situ analysis shows that BrdU-positive cells are also positive for DNA fragmentation. These results suggest that en route to apoptosis, cells undergo events typical of early cell-cycle traverse by expressing early $G_1$ genes and may even experience the late $G_1$/S phase boundary, as shown by the presence of PCNA. However, the demonstrated ability of these cells to traverse the $G_1$ phase of the cell cycle seems to be an abortive event, since they die shortly afterwards. 

**Key words:** cell cycle, cell death, apoptosis, Swiss 3T3 cells, DNA fragmentation

Apoptosis is an active cellular process of gene-directed self-destruction [Kerr et al., 1972]. This phenomenon has been shown to be necessary to normal developmental and homeostatic processes [Kerr and Harmon, 1991]. Programmed cell death is readily apparent during metamorphosis in insects and amphibians [Hurte et al., 1981], during neuronal development [Oppenheim, 1991; Barres et al., 1992], in the immune system in mammals [Goldstein et al., 1991; Cohen et al., 1992], and during development of *C. elegans* [Yuan and Horvitz, 1990]. Cell death through apoptosis is distinct from pathological cell death (i.e., necrosis); necrosis involves disruption of membrane integrity and subsequent cellular swelling and lysis [Trump et al., 1984], while apoptosis is characterized by membrane blebbing, chromatin condensation, and DNA fragmentation into oligonucleosomal fragments giving a characteristic ladder pattern on ethidium bromide–stained agarose gels [Kerr and Harmon, 1991].

Several models of in vitro studies of apoptosis have been described, in which programmed cell death can be induced in different cell types in culture by a number of stimuli, such as introduction or deprivation of hormones and lymphokines in lymphocytes or growth factor deprivation in transformed fibroblasts. From studies on apoptosis in in vitro systems and genetic analysis of cell death during development of the nematode *C. elegans,* a number of genes have been shown to be involved in this process. Expression of an early cell-cycle gene, *c-myc,* upon serum deprivation has been shown to induce apoptosis in rat fibroblasts [Evan et al., 1992], whereas

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expression of the *bcl-2* oncogene counteracts this effect of *c-myc* and rescues the cells from apoptosis under similar conditions [Bissonnette et al., 1992; Fanidi et al., 1992]. In *C. elegans* two genes, *ced-3* and *ced-4,* have been shown to be required for cell death during development [Yuan and Horovitz, 1990]; expression of *ced-9* seems to antagonize the effects of *ced-3* and *ced-4* [Hengartner et al., 1992]. Recent studies indicate that *ced-9* is a homologue of the mammalian *bcl-2* oncogene, whose expression is linked to the inhibition of apoptosis [Vaux et al., 1992]. In spite of these findings, the actual mechanism determining the process of apoptosis is still far from being understood. The cascade of signal transduction events, from the plasma membrane to the nuclear events involved in the process leading to apoptosis, is yet to be dissected.

We have studied the programmed cell death of density-arrested Swiss 3T3 cells, induced by serum deprivation. Density-arrested quiescent Balb-c 3T3 cells are dependent upon growth factors for their survival and die within 5–10 h after withdrawal of growth factor–containing serum from the medium [Tamm and Kikuchi, 1990]. However, the mechanism of this cell death is not known. We report here that the death of density-arrested Swiss 3T3 cells induced by serum deprivation is through apoptosis, as is evident from the changes in nuclear morphology and DNA fragmentation analysis of dying cells and the early reversibility of the process. We further present evidence which indicates that apoptosis in this condition is accompanied by expression of early cell-cycle genes and that, following this event, cells even reach the $G_1$/S border, as indicated by the presence of proliferative cell nuclear antigen (PCNA). In addition, a noticeable level of BrdU is observed, possibly reflecting the synthesis of DNA involved in repair activity. This is followed by cell death, as shown by in situ DNA fragmentation analysis. These results thus show that mechanistically, upregulation of early cell-cycle gene expressions such as *c-fos, c-myc, c-jun, cdc2,* and RB phosphorylation and the experience of some part of the $G_1$ phase of the cell-cycle traverse are involved in the process of programmed cell death. This notable experience is an abortive one, since it is followed by subsequent death.

## GENERAL TECHNIQUES FOR STUDYING PROGRAMMED CELL DEATH AND EARLY CELL-CYCLE TRAVERSE

### Cell Culture Techniques

The easiest in vitro model for studying programmed cell death is the withdrawal of serum from cultured mouse 3T3 fibroblasts. We have cultured Swiss 3T3 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. Approximately $5 \times 10^5$ cells were seeded into 100 mm petri dishes; cells were grown at 37°C in an incubator maintained at 7.5% $CO_2$. When cultures reached 100% confluency, they were left in the quiescent state for an additional 24 h to ensure a complete absence of DNA synthesis. For serum deprivation experiments, the monolayer cultures containing these density-arrested cells were washed twice with serum-free DMEM and incubated thereafter in the same serum-free DMEM. In control experiments, medium was replaced with fresh DMEM containing 10% FBS. For the experiments studying the effect of inhibitors of protein and RNA synthesis on cell survival in serum deprivation conditions, density-arrested cells were pretreated for 1 h with DMEM containing 1.0 mM cycloheximide or cytosine-β-D-arabino-furanoside (Ara-c) (both obtained from Sigma Chemical Company, St. Louis, MO), followed by washing with serum-free DMEM and further incubation with serum-free DMEM containing the same amount of cycloheximide or Ara-c. Cells were harvested at different time points, and viable cells were counted by trypan-blue exclusion assay.

### Protein Extraction and Immunoblot Analyses

Cells were harvested at indicated time points after total removal of serum in cold (4°C) phosphate-buffered saline (PBS) by scraping and further pelleted by centrifugation at 1,000*g*. The cell pellet was washed once with cold PBS and then resuspended in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA at pH 7.4 containing 0.5 mM phenylmethylsulfonylfluoride (Sigma), 10 μg/ml aprotinin, and 2 μg/ml each of pepstatin and leupeptin [Boehringer-Mannheim, Mannheim, Germany]). These cell pellets were collected, resuspended at a concentration of $3 \times 10^6$ cells/ml, and sonicated in an ice bath for 5 min. These

cell extracts were then spun at 5,000*g* for 10 min; the supernatant was collected as the source of total protein.

One hundred micrograms of total protein for each sample was separated on SDS/PAGE (10% gels to detect *c-myc, c-fos,* and *c-jun,* 14% for *cdc2,* and 7.5% for RB gene products) and transferred onto a nitrocellulose membrane. Blots were further incubated in Tris-buffered saline (TBS) (10 mM Tris-HCl (pH 7.5), 300 mM NaCl) containing 0.5% Tween-20 for 1 h and then transferred to solutions containing various primary antibodies (mouse monoclonal antibody to *cdc2* and rabbit polyclonal antibody to c-*jun* protein were obtained from Oncogene Science Inc. (Saranac Lake, NY); mouse monoclonal antibody to c-*myc* and rabbit polyclonal antibody to c-*fos* from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); and mouse monoclonal antibody to retinoblastoma protein (RB) from Pharmingen [San Diego, CA] diluted in TBS containing 0.05% Tween-20 to the working concentration and incubated at 4°C for 14 h, followed by washing with TBS containing 0.5% Tween-20. Blots were then incubated with either rabbit antimouse immunoglobulin G (IgG) (in the case of mouse monoclonal primary antibodies) or goat antirabbit IgG (for rabbit polyclonal antibodies) and then conjugated to alkaline phosphatase for 1 h at room temperature, after which blots were washed; positive antibody reaction was reflected by the blue colour developed using nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BICP).

### Immunofluorescence Microscopy

Swiss 3T3 cells were grown on poly-L-lysine–coated coverslips and subjected to serum deprivation as described above. At different time points after serum withdrawal, cells were fixed with acetone:methanol (1:1) at −20°C for 10 min. Coverslips were then washed with phosphate-buffered saline (PBS) and incubated with polyclonal antibody to c-*fos* (Santa Cruz Biotechnology Inc.) or monoclonal antibody to PCNA (Coulter Diagnostics, Miami, FL) diluted 1:100 with PBS, overnight at 4°C. Coverslips were then washed four times with PBS, incubated with rabbit antimouse IgG (this step was omitted in the case of rabbit polyclonal anti-c-*fos* antibody) for 1 h at room temperature, washed, and incubated with goat antirabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC) for 1 h at room temperature, washed again, mounted in 50% glycerol, and examined under a Nikon Labophot fluorescence microscope equipped with epiillumination capability. Secondary and tertiary antibodies were purchased from Cappel Research Reagents (Turnholt, Belgium). Whole nuclear Hoechst staining was accomplished by adding 0.5 μg/ml bisbenzimide (M33258; Boehringer-Mannheim, Mannheim, Germany) to the last wash solution.

### DNA Extraction and Agarose Gel Analysis

Cells were harvested and washed in PBS, and the cell pellet was resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, RNAse A (20 μg/ml), and 0.5% SDS. The cell extracts were incubated at 37°C for 1 h, after which proteinase K (Boehringer-Mannheim) was added to a final concentration of 100 μg/ml, and digestion was continued for another 3 h at 50°C. After digestion, the reaction mixture was extracted three times with phenol saturated with 100 mM Tris-HCl buffer (pH 7.4); DNA was precipitated from the aqueous phase by ammonium acetate and ethanol at −20°C. The precipitate was washed with 75% ethanol and finally dissolved in Tris-EDTA (TAE) buffer (10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA). DNA (10 μg) from each sample was analysed on a 1% agarose gel containing ethidium bromide by electrophoresis in TAE buffer at 20 volts for 14 h at room temperature; to increase detection sensitivity, the gel was further incubated in a 50 mg/l ethidium bromide solution.

### In Situ DNA Degradation Assay

Swiss 3T3 cells were grown on poly-L-lysine–coated glass coverslips and subjected to serum deprivation as described above. At different time points after serum deprivation, cells were fixed with acetone:methanol (1:1) at −20°C for 10 min. Analysis of DNA fragmentation was done using DNA nick end-labelling by terminal deoxynucleotidyl transferase (TdT) (from Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada), as described by Gavrieli et al. [1992]. Fixed cells on coverslips were rehydrated in PBS for 30 min and then washed twice with double-distilled water (DDW), after which the coverslips were covered with TdT reaction buffer (30 mM Trizma base (pH 6.8), 100 mM sodium cocadylate, 5 mM cobalt chloride, 0.5 mM dithiothreitol, 0.1 mg/ml

bovine serum albumin) containing TdT (0.3 unit/μl) and biotin-16-UTP (from Boehringer-Mannheim) at 10 μM final concentration. The reaction was carried out at 37°C for 1 h in a humidified chamber and terminated by transferring the coverslips to a solution containing 300 mM NaCl and 30 mM sodium citrate for 15 min at room temperature, after which the coverslips were rinsed in double-distilled water and incubated in 2% BSA for 10 min, followed by incubation with 20 μg/ml fluorescein-conjugated streptavidin (Boehringer-Mannheim) in PBS for 30 min at room temperature; they were then washed twice in PBS and once with distilled water, mounted in PBS containing 50% glycerol, and examined with a Nikon fluorescence microscope or Bio-Rad MRC600 Confocal microscope.

### BrdU Incorporation Assay

Cells engaged in DNA synthesis activity can be identified by the incorporation of bromodeoxyuridine (BrdU), which can be detected with anti-BrdU antibody followed by indirect immunofluorescence assays. Cells were grown on poly-L-lysine–coated coverslips and subjected to serum deprivation as described above, except that BrdU was added to the medium to a final concentration of 10 μM 3 h before fixing the cells in cold acetone/methanol. Fixed cells were treated with 2 M HCl at room temperature for 30 min and then washed with PBS three times, 15 min for each wash. Coverslips were then incubated with anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry System, Mountain View, CA) for 1 h at room temperature, washed with PBS as before, and then incubated with rabbit anti-mouse immunoglobulin G (obtained from Cappel Research Reagents) at room temperature for 1 h, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated goat antirabbit IgG for 30 min, followed by washing and mounting in 50% glycerol. For double-labelling experiments involving detection of BrdU incorporation and DNA fragmentation, coverslips were first subjected to the end-labelling reaction described above and then to indirect immunofluorescence assay using anti-BrdU antibody and rhodamine-conjugated goat anti-mouse IgG (from Cappel Research Reagents). The coverslips were analysed using the Bio-Rad MRC600 Confocal image analysis system.

### [$^3$H]-Thymidine Incorporation Assay

Cells were grown on six-well microtitre plates until they reached 100% confluency, further kept at this density-arrested state for an additional 24 h, and then subjected to serum deprivation as described above. [$^3$H]-thymidine (ICN Radiochemicals, Mississauga, Ontario) was added to the medium to a final concentration of 10 μCi/ml, and cultures were incubated for 1 h before processing the cells for scintillation counting. In brief, after 1 h cells were washed with cold PBS three times and treated with 10% trichloroacetic acid (TCA) at 0°C for 10 min, and the precipitates were washed with PBS. The TCA-precipitable fraction was solubilized in 0.2 N NaOH at 65°C and neutralized with 0.2 N HCl. This solution was aliquotted to appropriate fractions for use in liquid scintillation counting.

## DENSITY-ARRESTED SWISS 3T3 CELLS UNDERGO APOPTOSIS WHEN DEPRIVED OF GROWTH FACTORS

Density-arrested Balb/c-3T3 cells have been shown to depend on growth factors for their survival [Tamm et al. 1991; Tamm and Kikuchi, 1990]; these cells die rapidly when deprived of serum. We studied the behavior of density-arrested Swiss 3T3 cells under conditions of serum deprivation; results of a viability assay, as determined by trypan blue exclusion detection, are shown in Figure 1. When subjected to serum deprivation, these cells started dying rapidly, about 20% by 6 h and about 40% by 12 h after serum deprivation; after 48 h of serum deprivation, only 25% of the cells remained viable. Inclusion of either the protein synthesis inhibitor cycloheximide or the RNA synthesis inhibitor cytosine-β-D-arabino-furanoside (Ara-c) at 1 μM concentration in serum-free medium in serum deprivation experiments delayed the extent of cell death by 24 h, as seen by comparing the proportion of viable cells at 12 h in the control serum-deprived population with that at 36 h in the drug-treated sample, suggesting the involvement of an active cell death mechanism.

To determine whether the cell death in such conditions is due to necrosis or a physiologically active death program, we examined the apoptosis by unique cellular and nuclear morphology and found a characteristic pattern of DNA fragmentation resulting from the cleavage of nuclear DNA in internucleosomal regions. Under a phase

contrast microscope, the dying cells displayed the characteristic features of apoptosis, such as blebbing of plasma membrane, as well as the appearance of smaller refractile apoptotic bodies (Fig. 2A–C). Nuclear staining of the cells, fixed at different times after serum deprivation with propidium iodide staining, is shown in Figure 3A–C. Condensed chromatin and nuclear fragmentation are seen clearly in dying cells after 6 and 18 h of serum deprivation (Fig. 3B,C). The number of nuclei with features similar to apoptosis increases with time after serum deprivation and reaches a maximum by 24 h.

Furthermore, when DNA isolated from cells at different time points after serum deprivation was analysed on an ethidium bromide–stained agarose gel, we observed the typical ladder pattern of DNA fragmentation (Fig. 4). DNA degradation into oligonucleosomal fragments begins to be visible after 6 h of serum deprivation; the extent of DNA fragmentation increases up to 24 h after serum deprivation. Since in this assay the DNA was isolated from total cells, including both the dying and viable subpopulations, the increase in the DNA fragmentation seen on an agarose gel is probably due to assaying with mass cultures, which measures the increase in both the number of dying cells and the extent of fragmentation in each cell. Recently a method has been developed to observe DNA fragmentation in fixed individual cells by in situ labelling of the 3′-end of fragmented DNA, using terminal deoxynucleotidyl transferase and biotinylated UTP [Gavrieli et al., 1992]; we used this method to demonstrate in situ DNA fragmentation with minor modifications. Swiss 3T3 cells were grown on poly-L-lysine–coated coverslips, fixed at different times after serum deprivation,

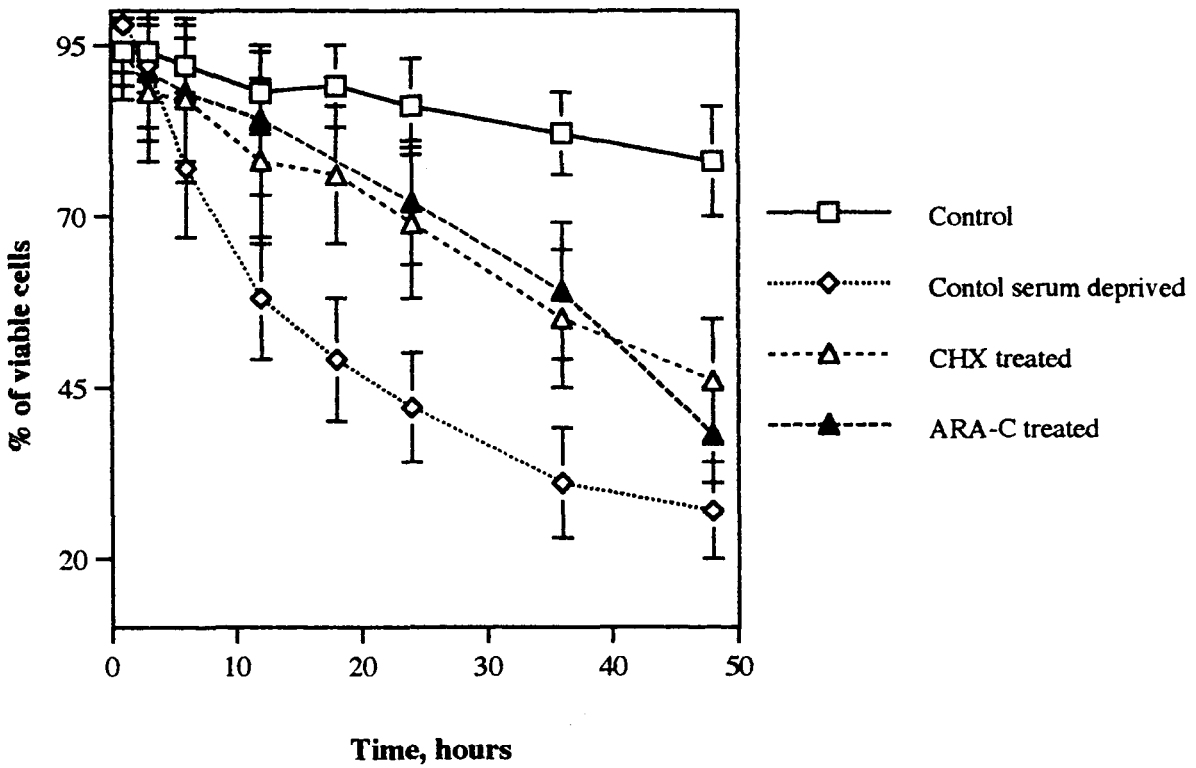


**Fig. 1.** The survival curve of density-arrested quiescent Swiss 3T3 cells during serum deprivation. Swiss 3T3 fibroblasts were grown in a six-well microtitre plate to 100% confluency, density-arrested for an additional 24 h, and subjected to serum deprivation. Viability at each time point was determined by trypan blue exclusion. Cells were harvested in phosphate-buffered saline (PBS); 10 μl cell suspension was mixed with 10 ml of trypan blue (0.4% stock from GIBCO-BRL Life Technologies [Québec City, Québec, Canada]) and incubated for 5 min, and cells excluding the dye were counted in a hemocytometer. The results represent the mean value obtained from four independent sets of experiments; the bars indicate the upper limit of one standard deviation at each time point. Viability is shown in control medium containing 10% fetal bovine serum (FBS) (open squares); in the absence of serum (i.e., serum-deprived) without any inhibitor (open diamonds); in serum-free medium with 1 mM protein synthesis inhibitor, cycloheximide (CHX, open triangles) or RNA synthesis inhibitor, cytosine-β-D-arabinofuranoside [Ara-c] (solid triangles). Inhibitors were added 1 h before serum withdrawal.

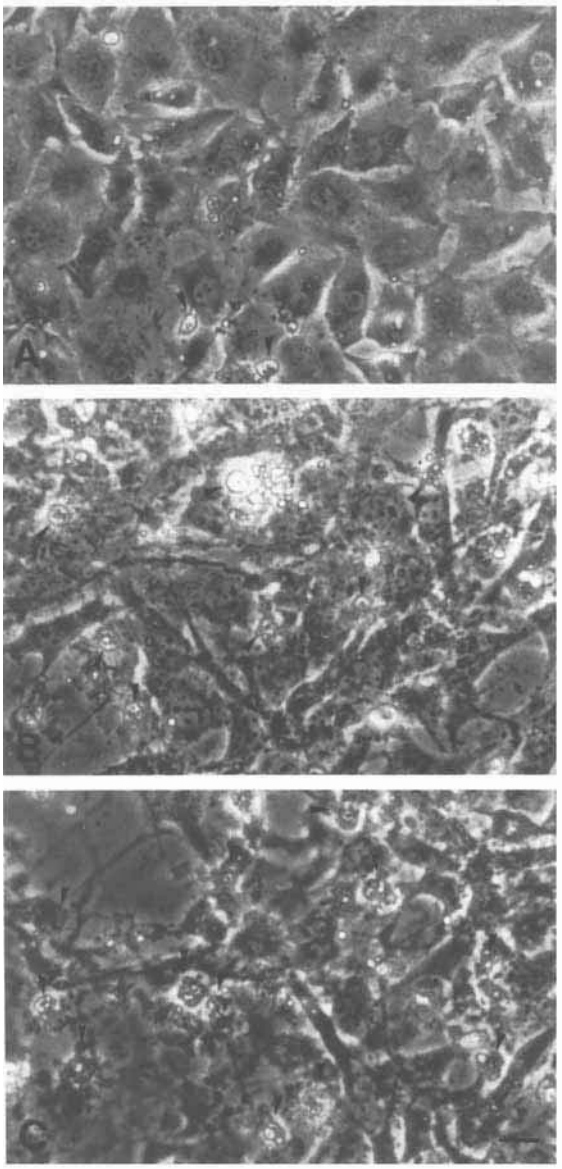

Fig. 2. Cell morphology during serum deprivation of density-arrested Swiss 3T3 cells. Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, density-arrested for 24 h, and subjected to serum deprivation. Cells were photographed at different time points (0, 6, and 18 h shown in **A,B,C,** respectively) after serum deprivation using a Nikon inverted microscope. Arrowheads are used here to indicate the apoptosis-associated effect: refractile bodies in the monolayer culture. Bar = 25 μm.

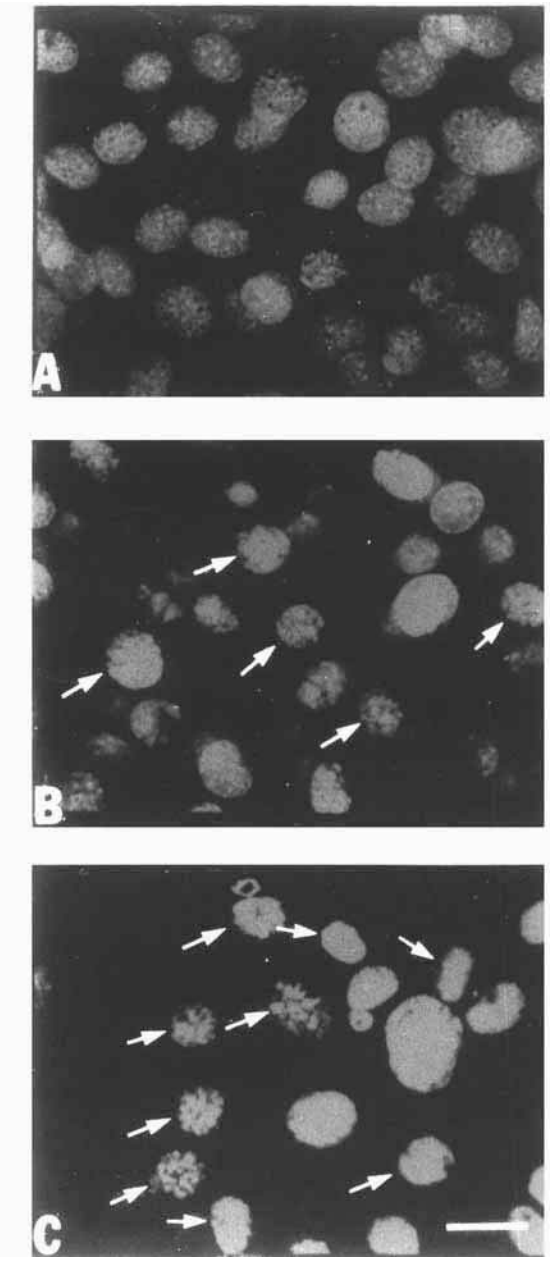

Fig. 3. Morphology of the nuclei of cells at different time points after serum deprivation. Cells were grown on poly-L-lysine–coated glass coverslips, density-arrested for 24 h, and subjected to serum deprivation. Cells were fixed at 0, 6, and 18 h after serum deprivation (**A,B,C,** respectively) in methanol: acetone at −20°C for 5 min; after air drying the specimens were washed with PBS and stained with propidium iodide diluted in PBS at a concentration of 1 μg/ml (from ICN Biomedicals). Coverslips were then washed in PBS and double-distilled water, mounted in 50% glycerol, and analysed in a Bio-Rad MRC600 Confocal image analyser. Arrows are used here to identify cells showing typical apoptosis-associated chromatin condensation and nuclear fragmentation. Bar = 25 μm.

and then subjected to an end-labelling reaction; results are shown in Figure 5. Almost all the control cells are negative for DNA fragmentation (Fig. 5A); the number of cells showing DNA fragmentation increases with time after serum deprivation, and by 18 h after serum withdrawal, more than 80% of the cells show intense staining for DNA fragmentation. These results

are similar to the kinetics of cell death by DNA fragmentation observed in DNA ladder assays by agarose gel electrophoresis (Fig. 4) and precede the kinetics of the viability test (Fig. 1). Interestingly, we find that the advantage of the in situ fragmentation assay is that one can measure the cell death event on an individual cell basis; this is especially important when the incidence of cell death is of too low a magnitude to be detected by the mass culture DNA ladder assay. Thus, taken together, the morphological features and DNA fragmentation analysis clearly demonstrate that the death of density-arrested Swiss 3T3 fibroblasts under serum deprivation occurs through the pathway defined as programmed cell death (apoptosis).

## EARLY CELL-CYCLE-SPECIFIC GENES ARE EXPRESSED DURING APOPTOSIS

Involvement of an early cell-cycle-specific gene, *c-myc*, has been shown recently in apoptosis of rat fibroblasts under conditions of growth factor deprivation [Evan et al., 1992]. In order to investigate whether *c-myc* and other cell-cycle-specific genes are involved in serum-deprivation-induced apoptosis in our system of density-arrested Swiss 3T3 cells, we studied the expression of these genes at the protein level at different times after serum deprivation. Total protein was extracted from the cells at different time points after serum deprivation, and protein samples of equal size (100 μg) were separated on SDS-PAGE; the separated proteins were then electrotransferred to nitrocellulose filter blots. Blots were subjected to Western analysis using

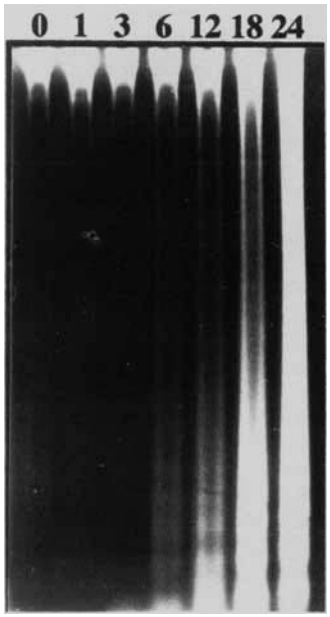


**Fig. 4.** DNA fragmentation following serum deprivation of density-arrested Swiss 3T3 cells. DNA was extracted from the density-arrested Swiss 3T3 cells at 0, 1, 3, 6, 12, 18, and 24 h after serum withdrawal; time of serum deprivation is indicated above each lane. DNA (10 μg) from each time point sample was separated electrophoretically on 1% agarose gel and visualized by UV after staining with ethidium bromide.

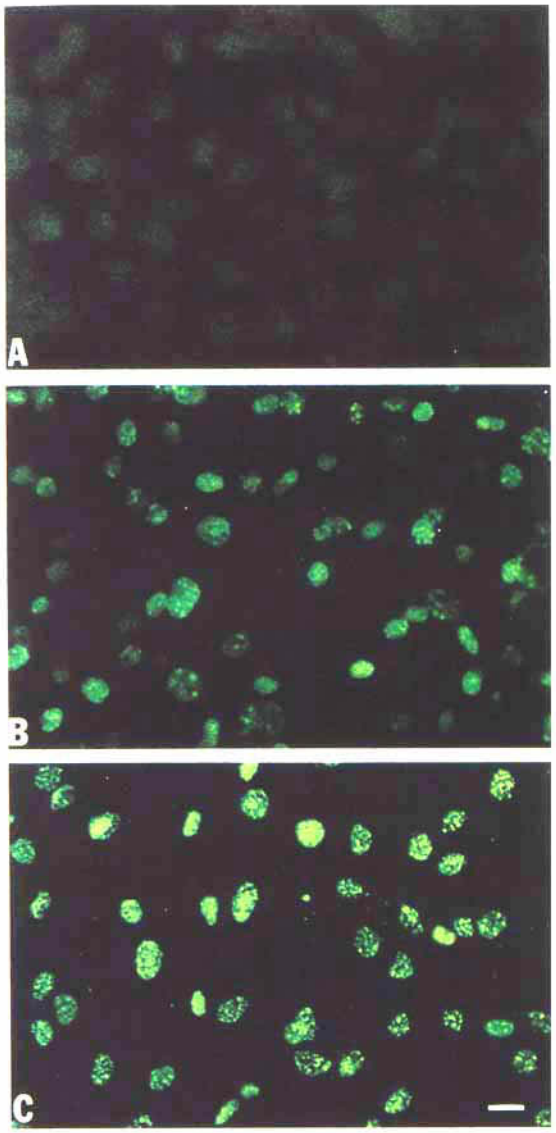


**Fig. 5.** In situ DNA fragmentation analysis. Cells were grown on coverslips, subjected to serum deprivation, and fixed at 0, 6, and 18 h after serum deprivation (**A,B,C,** respectively). Fixed cells were subjected to 3'-end labelling reaction using terminal deoxynucleotidyl transferase and biotin-16-UTP, followed by detection of incorporated biotin by streptavidin conjugated to fluorescein (for details see text section on General Techniques), and analysed under a Confocal image analysis system. Bar = 25 μm.

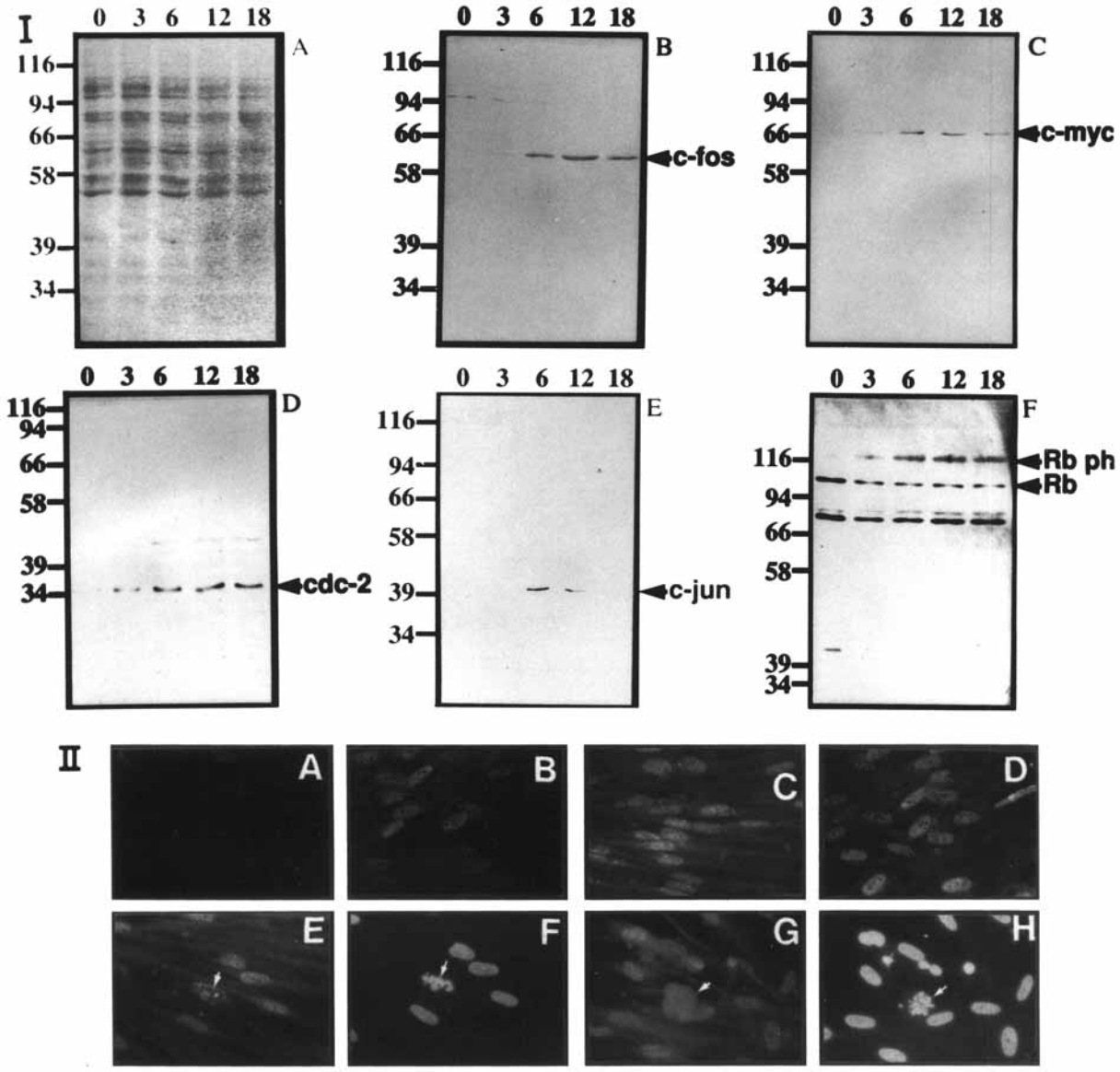


**Fig. 6.** Western blot and immunofluorescence analysis of expression of *c-fos, c-myc, cdc2, c-jun,* and RB at different time points after serum deprivation. **Panel I:** Protein (100 μg) from cell extracts from each time point was used in each lane; proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The blots were subjected to immunoblot analysis by incubating with antibodies to RB, *cdc2,* c-*myc* (monoclonal), c-*jun,* and c-*fos* (polyclonal) at 4°C overnight. Blots probed with polyclonal antibodies were washed and incubated with goat antirabbit IgG conjugated to alkaline phosphatase (Cappel Research Reagents) for 1 h and washed; positive reaction was developed using nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate. Those blots which were probed with monoclonal antibodies were washed, incubated first with goat antimouse IgG conjugated to alkaline phosphatase, and developed after washing as described above. Time of serum deprivation is indicated above each lane; the positions of molecular weight markers are indicated in the left margins. **A:** The general protein pattern as stained by Coomassie blue dye, showing the similarity among all time points in terms of the amount of protein loaded and the protein profile. **B:** The presence of c-*fos* beginning at 3 h, continuing to 12 h, with a slight decrease at 18 h. **C:** The presence of c-*myc,* appearing at 3 h, increasing at 6 and 12 h, and decreasing slightly at 18 h. **D:** The gradually increasing presence of *cdc2* from 3–18 h, similar to c-*fos.* **E:** c-*Jun* presence at 6 and 12 h. **F:** The change in RB phosphorylation, revealed by the detection of an additional band at 116 kDa (Rb ph) beginning at 3 h and increasing until 18 h, while the unphosphorylated p110 band (Rb) decreases in intensity throughout the time span. A lower molecular weight (80 kDa) band seen at all time points seems to be a nonspecific reaction and serves as an internal control for the standard of protein quantity loaded in each lane. **Panel II:** Cells were grown on coverslips, subjected to serum deprivation, and fixed at different time points after serum deprivation. Coverslips were incubated with anti-c-*fos* polyclonal antibody; the presence of the antigen was detected using goat antirabbit IgG antibody conjugated with fluorescein isothiocyanate. Time points displayed are 0, 3, 6, and 12 h for **A,B,C,D,** respectively; **E,F** (12 h) presents the same areas as **G,H** (18 h). E,G: c-*fos* antibody staining; nuclei are visualized by Hoechst stain in F,H. Arrows indicate fragmented nuclei, showing positive staining for c-*fos* antibody. Comparative staining intensity here reveals that some nuclei possess c-*fos* as early as 3 h; the number of c-*fos*–positive nuclei increases with time. Abnormal nuclear morphology such as fragmented chromosomes, observed at 12 (E,F) and 18 h (G,H), also corresponds with positive c-*fos* staining. ×820.

specific antibodies to *cdc2, c-myc, c-jun, c-fos,* and RB proteins.

As shown in Figure 6, *c-fos, c-myc, c-jun,* and *cdc2* protein presence is undetectable at the 0 h time point, when the cultures are kept in the quiescent state; however, visible bands showing their presences are clearly seen by 6 h after the initiation of programmed cell death by the removal of serum. In the case of c-*fos* (Fig. 6, Panel I, B), protein presence is detectable even as early as 3 h and continues at high levels till 18 h with only slight decrease. Similarly, c-*myc* is detected at 3, 6, and 12 h, but its presence decreases more than that of c-*fos* by 18 h (Fig. 6, Panel I, C). *cdc2* is also present in a similar pattern; and like c-*fos,* its level remains high till 18 h (Fig. 6, Panel I, D). c-*Jun* is only detected at 6 and 12 h; by 18 h, its presence is totally undetectable (Fig. 6, Panel I, E). Similar changes were also observed with RB phosphorylation: at 0 h only the 110 kDa unphosphorylated RB band was observed, while the 116 kDa phosphorylated band appeared as early as 3 h and intensified to outweigh the 110 kDa band by 18 h (Fig. 6, Panel I, F). To verify the cellular distribution of this upregulation of these early $G_1$ proteins en route to programmed cell death, we performed immunofluorescence staining for c-*fos* presence as a selected example. As shown in Figure 6, Panel II, the nuclear staining of c-*fos* is undetectable at time 0; it becomes noticeable in some cells by 3 h and increases till 12 h. By 12 and 18 h c-*fos* is not only present in significant amounts in intact cells, but is also seen in those nuclei exhibiting fragmented nuclear morphology (Fig. 6, Panel II, E–H).

## CELLS EXPERIENCE A SMALL DEGREE OF DNA SYNTHESIS ACTIVITY DURING APOPTOSIS

Results of the experiments described above clearly indicate the induction of early cell-cycle-specific genes soon after serum deprivation in density-arrested quiescent 3T3 cells. This observation raises the interesting question of whether the cells die at the $G_1$/S boundary or eventually enter the very early part of S phase, beyond the $G_1$ border, before dying. We address this question by two independent methods: radioactive thymidine and bromodeoxyuridine incorporation assays for in situ labelling of cells engaging in DNA synthesis activity. Initially, cells were labelled with [$^3$H]-thymidine at different time points after serum deprivation, and TCA-precipitable counts were taken as an index for DNA synthesis activity level. As shown in Figure 7, thymidine incorporation increases sharply at 3 h after serum deprivation and then decreases to a basal level at later time points.

This assay gives an overall incorporation of thymidine in a population of cells, but it does not give details regarding individual cells and thus cannot distinguish between incorporation by dying and normal cells. We examined individual cells at different time points after serum deprivation by in situ BrdU labelling. Cells were grown on coverslips and subjected to serum deprivation as described before, with the addition of bromodeoxyuridine to the culture at a final concentration of 10 μM at 3 h before fixation at designated time points. Fixed cells were subjected to indirect immunofluorescence assay using an anti-BrdU antibody and an appropriate second antibody. Cells were also stained with a DNA staining dye, propidium iodide, to indicate the total number and gross morphology of nuclei. As shown in Figure 8A, no nuclei are positive for BrdU staining at the zero time point, which is the density-arrested quiescent state. At 3 h after serum deprivation, a large proportion of total nuclei display positive BrdU incorpora-

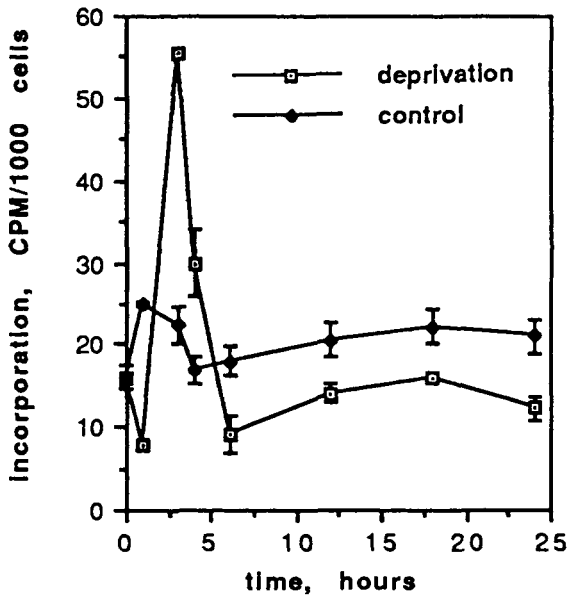


Fig. 7. Tritiated thymidine incorporation during apoptosis. Cells were labelled with tritiated thymidine for 1 h at different time points after serum deprivation, after which they were processed for liquid scintillation counting; TCA precipitable counts were plotted against time. The value at each time point is the mean of data obtained from four independent experiments; vertical bars indicate the upper limit of one standard deviation at each time point.

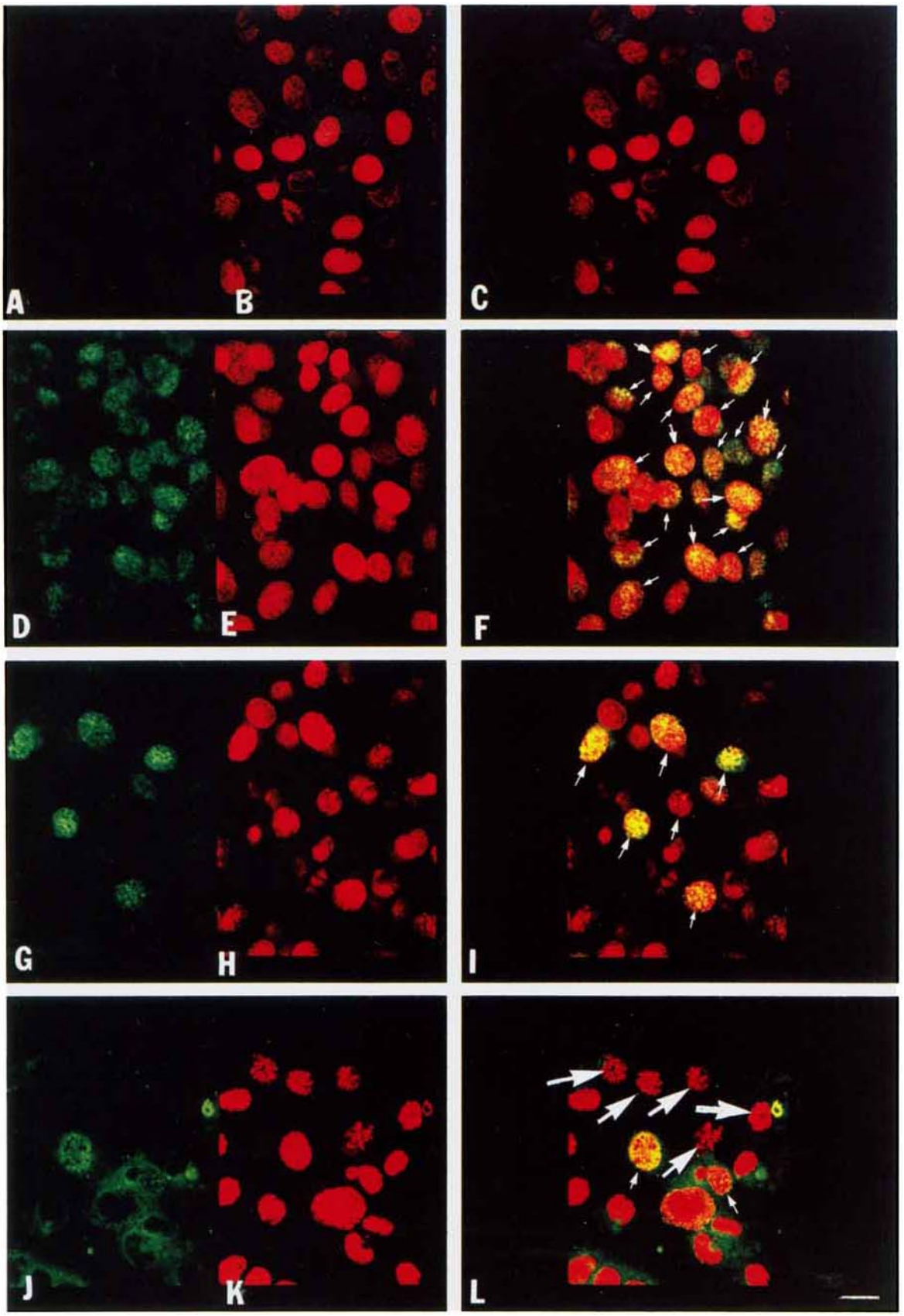


Fig. 8

tion. The number of positive cells decreases at later time points, but nevertheless approximately 20% of cells are BrdU-positive up to 18 h. These results are in agreement with the previous findings using thymidine incorporation.

Propidium iodide staining of nuclei at 18 h after serum deprivation (Fig. 8I,K,L) indicates that many nuclei clearly show nuclear condensation and fragmentation but are not labelled with BrdU (indicated by large arrows). This is due to the fact that the cells are labelled only for 3 h before the indicated time points, and only those cells which are synthesizing DNA at this time are labelled with BrdU. Therefore, the fragmented nuclei shown by propidium iodide staining must have undergone gross nuclear condensation and fragmentation before the BrdU pulse was given. However, there are many cells where BrdU-incorporated nuclei also show the characteristic features of apoptosis—that is nuclear condensation and fragmentation (Fig. 8G–L, indicated by small arrows).

In order to further confirm that cells are experiencing the $G_1$ phase of the cell-cycle traverse during this process, we examined the presence of another marker for this phase. Proliferating cell nuclear antigen (PCNA), shown to be DNA polymerase δ, is synthesized during mid and late $G_1$ phase and continues to be present throughout the rest of the cell cycle [Baptist et al., 1993; Bravo, 1986; Kill et al., 1991; Lee and Hurwitz, 1990]. We performed an indirect immunofluorescence assay using anti-PCNA antibody to determine the level of PCNA at different time points after serum deprivation. As shown in the results (Fig. 9), we find that the typical staining of PCNA-positive nuclei appears at approximately 2 h after serum deprivation, and by 3 h many cells in the culture are positive (Fig. 9B). A significantly high proportion of cells is positive for PCNA staining at 6 h after serum deprivation, indicating that cells are entering the cell cycle at slightly different times (Fig. 9C). This observation confirms in part the concatenation of molecular events involved in the $G_1$ phase of cell-cycle traverse during apoptosis and supports the fact that BrdU incorporation during

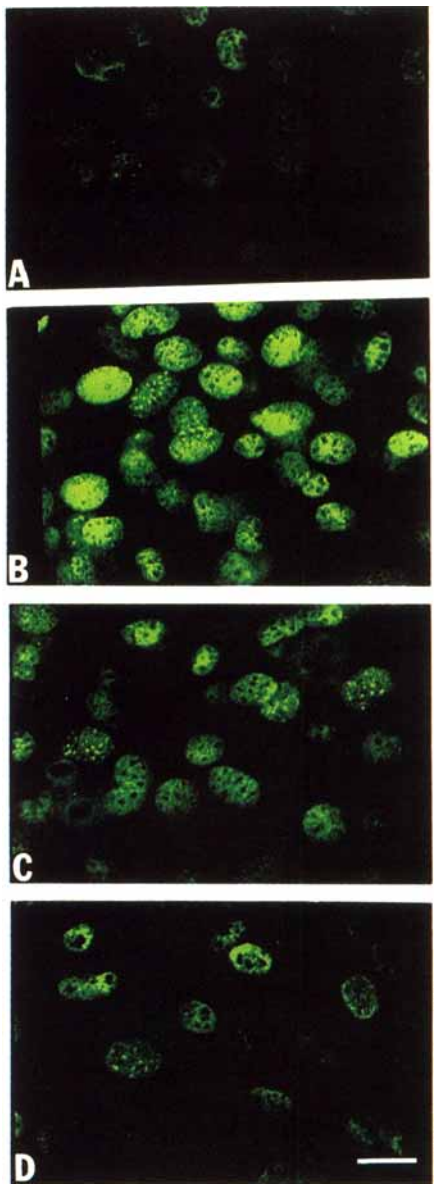


**Fig. 8.** BrdU incorporation assay in density-arrested Swiss 3T3 cells after serum deprivation. Cells were labelled with BrdU for 3 h before fixing at each time point after serum deprivation and subjected to indirect immunofluorescence staining using anti-BrdU antibody, as described in General Techniques. Cells were also stained with propidium iodide to show the total number of nuclei in the field, as well as chromatin condensation and nuclear morphology. **A,D,G,J:** BrdU labelling (green) at 0, 3, 6, and 18 h, respectively, after serum deprivation. **B,E,H,K:** Propidium iodide staining (red) of the same field as in A,D,G,J. **C,F,I,L:** The merged pictures of the red and green, producing orange- or yellow-coloured nuclei which are BrdU labelled (small arrows); large arrows indicate fragmented nuclei. Bar = 25 μm.

**Fig. 9.** Immunostaining of PCNA in cells after serum deprivation. Cells were grown on coverslips and fixed at 0, 3, 6, and 18 h after serum deprivation (**A,B,C,D,** respectively), incubated with mouse antiproliferating cell nuclear antigen (PCNA) antibody at room temperature overnight, washed, incubated with rabbit antimouse IgG followed by FITC-conjugated goat antirabbit IgG, and analysed under the Confocal microscope. PCNA-positive nuclei were most abundant at 3 and 6 h time points and decreased significantly at 18 h. Bar = 25 μm.

programmed cell death may be in part due to the synthesis of DNA. At later time points (18 h) (Fig. 9D), however, most cells are dead, and the number of PCNA-positive nuclei is likewise decreased.

Now it becomes interesting to ask whether DNA synthesis and DNA fragmentation are occurring at the same time in the same cells. To address this question, we performed a double-labelling experiment for in situ detection of DNA fragmentation and BrdU incorporation. BrdU-labelled cells were fixed and subjected to an end-labelling reaction as described above. After the reaction was over, the incorporated biotinylated uridine at the 3′-end of fragmented DNA was detected by a fluorescein-streptiavidin conjugate, and incorporated BrdU was detected by mouse anti-BrdU antibody and rhodamine-conjugated goat antimouse IgG. As shown in Figure 10, we did indeed observe colocalization of both activities (i.e., BrdU incorporation and DNA fragmentation) in some cells; the arrowheads indicate orange-coloured nuclei in Figure 10B–F, resulting from the coincidence of red and green labelling colours for rhodamine and fluorescein. It is noteworthy that not all the cells which are positive for DNA synthesis show DNA fragmentation staining (indicated by the large

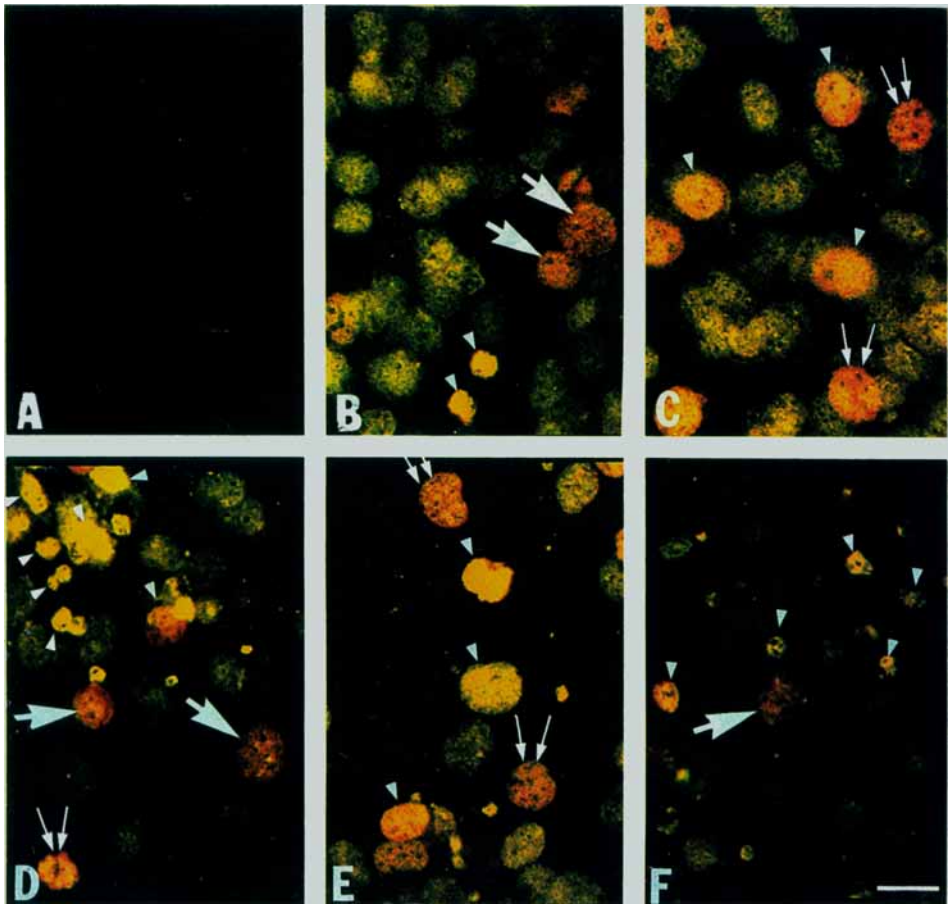


**Fig. 10.** Double-labelling for BrdU incorporation and DNA fragmentation during apoptosis of density-arrested Swiss 3T3 cells induced by serum deprivation. Cells were grown on coverslips and labelled with BrdU for 3 h before fixing at 0, 3, 6, 12, 18, and 24 h (**A–F**) after serum deprivation. Fixed cells were subjected to 3′-end labelling reaction using terminal deoxynucleotidyl transferase and biotin-16-UTP. Coverslips were then incubated with anti-BrdU antibody, washed in PBS, and incubated with rhodamine-conjugated goat antimouse IgG, followed by washing and further incubation with fluorescein-streptavidin conjugate. Coverslips were washed in PBS, mounted in 50% glycerol, and analysed under the Confocal image analysis system. Double-labelled nuclei are seen as yellow-coloured, due to the coincidence of green and red fluorescence; these cells are identified by arrowheads. However, there are cells (large arrows) showing only BrdU staining, seen in red only. The faint green-stained nuclei seen mostly in B and C are background staining of 3′-end labelling; double arrows point out those borderline cells showing positive BrdU staining throughout nuclei, while part of the nuclei show the orange colour reflecting localized initial DNA fragmentation. Bar = 25 μm.

arrow in Fig. 10B–F), implying that in these cells the degree of DNA fragmentation is below the sensitivity threshold of our assay methodology. Also, there are borderline cases with strong BrdU incorporation activity and initial detectable DNA fragmentation; in these cells, the red color representing the BrdU activity is very intense, while the green color representing the DNA fragmentation is less so. When the two colors coincide, the results show the red color as more pronounced, as indicated by double arrows in Figure 10C–E. These results suggest that a significant fraction of density-arrested quiescent Swiss 3T3 cells synthesize DNA during serum-deprivation-induced apoptosis, and this activity may coincide with the initiation of DNA fragmentation.

## CONCLUDING REMARKS AND PROSPECTIVES

The results presented in this paper demonstrate that density-arrested Swiss 3T3 cells die via the pathway of programmed cell death (apoptosis) upon serum deprivation. This is evident by the appearance of nuclear condensation as observed with propidium iodide staining and by fragmentation of DNA into oligonucleosomal fragments as shown by the characteristic DNA ladder pattern. DNA breakage was also analysed by in situ DNA fragmentation assay after serum deprivation of density-arrested Swiss 3T3 cells, by labelling the 3′-end of fragmented DNA using terminal deoxynucleotidyl transferase, as described by Gavrieli et al. [1992]. The serum-deprivation-induced death of density-arrested Swiss 3T3 cells in our experiments shows all the characteristic features of apoptosis. Furthermore, we also find that in the presence of protein or RNA synthesis inhibitors (cyclo-heximide or Ara-c), the extent of cell death is delayed by 24 h, indicating that it is an active process requiring the presence of newly synthesized proteins. Many cell types undergo apoptosis when they are deprived of serum or specific growth factors [Raff, 1992]. Survival of developing neurons depends upon neurotrophic factors secreted by neighbouring cells, in the absence of which they undergo apoptosis [Barres et al., 1992]. Activation of a cell suicide mechanism upon serum deprivation in density-arrested Swiss 3T3 cells may be similar to these in vivo cell deaths and is a good in vitro model for the study of activation of apoptosis in quiescent cells.

Expression of early cell-cycle genes such as *c-myc* has been shown to be involved in the induction of apoptosis in rat fibroblasts [Evan et al., 1992; Smeyne et al., 1993]. In our experimental system, we find the induction of protein appearance of not only c-*myc* but also other early cell-cycle genes, including *c-fos, c-jun,* and *cdc2,* in Swiss 3T3 cells en route to apoptotic death. Induction of expression of *c-fos* and *c-jun* oncogenes has been demonstrated in IL-2– and IL-6–dependent myeloma cells after induction of apoptosis by interleukin deprivation [Collatta et al., 1992]. These authors have shown that antisense inhibition of *c-fos* and *c-jun* expression protects mouse myeloma cells from apoptosis induced by interleukin deprivation. Furthermore, in an in vivo study of hormone-regulated apoptosis in prostate cells, Colombel et al. [1992] have shown that apoptosis results from reentry of differentiated cells into a defective cell cycle. Our results are consistent with these findings, with the additional finding of *cdc2* and *c-jun* protein presence, and support the notion that these early cell-cycle genes, and even a few selected $G_1$ cell-cycle genes, may be expressed in cells undergoing the process of programmed cell death. Furthermore, our observation that the phosphorylated form of the retinoblastoma tumour suppressor protein (RB) appears after induction of apoptosis in density-arrested cells indicates that cells are released from the quiescent state and enter the cell cycle en route to apoptotic death. Recent studies with mice carrying a homozygous disruption of the RB gene have demonstrated extensive neuronal cell death in the brains at embryonic day 11.5 and beyond [Jacks et al., 1992; Lee Ey et al., 1992; Clarke et al., 1992]. It has been proposed that due to loss of RB function in transgenic mice, the neuronal cells are unable to cease proliferation and finally enter the programmed cell death pathway. The phosphorylation of RB protein after serum deprivation of density-arrested cells has been suggested as an important step needed to release cells from the cell-cycle block, required for their reentry into the cell cycle. Taken together, these observations demonstrate that in our system of density-arrested mouse fibroblasts, cells en route to apoptotic death exhibit molecular events similar to those observed in the $G_1$ phase of the normal cell cycle. It seems that these cells, upon induction of apoptosis, are able to depart from the quiescent state and experience some part of the $G_1$ phase of the cell cycle. However, this

entrance should be noted as abortive, since the end result is programmed cell death rather than cell division, as normally seen in cell-cycle traverse.

Transgenic mice expressing T-antigen in postmitotic cerebellar Purkinje cells develop a typical cerebellar disease due to T-antigen dose-dependent cell death [Fedderson et al., 1992]; it has been postulated that the death of these cells results from stimulation of cell-cycle events. Similarly, induction of apoptosis by E1A transfection has been demonstrated in which mutant E1A, defective in induction of the complete cell-cycle traverse, is also defective in induction of apoptosis in transfected cells [Stein et al., 1991; White et al., 1991]. Thus, the failure of mutant E1A to induce cell-cycle traverse is correlated with a similar failure to induce apoptosis [Debbas and White, 1993]. Recently it has been shown that apoptotic prostate epithelial cells actively enter the cell cycle while dying [Colombel et al., 1992]. Here, our results represent strong evidence that cells do indeed enter the $G_1$ phase of the cell cycle during apoptosis, by the direct demonstration of early $G_1$ phase proteins in dying cells after serum deprivation.

Surprising but interesting is the observation that a large proportion of density-arrested cells show thymidine incorporation at approximately 3 h after serum deprivation. The normal induction of cells by mitogen from $G_0$ to S phase takes approximately 8–10 h; the shortening of this time for DNA synthesis activity during serum deprivation suggests that this activity may constitute an attempt at DNA repair. As shown in Figures 4 and 5, as early as 6 h after the activation of apoptosis by serum deprivation, there is already notable damage to DNA integrity. It is entirely possible that cells activate their repair machinery in response to this damage; however, the attempt is too feeble to overcome the magnitude of the DNA damage and eventually succumbs to death. Nevertheless, we do not rule out the possibility that incorporation of BrdU or thymidine may be due to DNA synthesis and not to endogenous terminal transferase activity from DNA repair, since we also observe the appearance of significantly elevated PCNA levels after serum deprivation, in correlation with BrdU incorporation. Elevated PCNA is not usually associated with DNA repair; thus, the significance of this protein's presence requires further investigation. We show here the elevation of a single member of the DNA synthesis machinery: PCNA gene expression. The definitive answer as to how DNA repair is regulated, and what the role of PCNA is in this process, requires extensive biochemical study of other molecules involved in DNA synthesis in general, such as examination of DNA polymerases including $\alpha$, $\beta$, and $\gamma$ as well as topoisomerase and helicase activity. Detailed studies of this aspect are in our future study plans.

Another point of interest is that the presence of c-*fos*, c-*myc*, c-*jun*, *cdc2*, and RB phosphorylation peaks by 12 h after activation of apoptosis, whereas DNA fragmentation (as shown in Fig. 4) is maximal at 18 h. This delayed pattern of DNA fragmentation, after the maximal level of early cell-cycle gene products is attained, suggests a possible relationship between the two events. Might there exist a cascade pathway leading to apoptotic death, with serum deprivation as an initial triggering action, subsequently inducing the activation of early cell-cycle genes, followed by maximal mass DNA fragmentation? If such a pathway exists, it resembles closely the signal transduction pathway stimulated by mitogen, except that the initial triggering is accomplished by withdrawing, rather than adding, serum, and the end result is DNA fragmentation, rather than successful DNA replication. Where the two pathways—that is, apoptosis vs. replication—diverge, and what are the regulators dictating the divergence between the two, will be the focus of much intense research in the near future.

The combination of c-*fos* and c-*jun* to form the AP-1 complex, a strong transcription factor, is the cellular event which is most probably essential to the molecular machinery of apoptosis. We noticed that both c-*fos* and c-*jun* are detectable at 6–12 h after serum deprivation; the presence of this AP-1 complex may reflect a functional role in activating putative downstream genes involved in later apoptotic events. The antibody we used to detect c-*jun* does not crossreact with the *Jun-B* protooncogene product; since c-*fos* can form the AP-1 complex with either c-*jun* or *Jun-B*, the absence of c-*jun* at the later time point may indicate its replacement by *Jun-B* in the complex. Once specific antibody to *Jun-B* becomes available to us, we will examine this possibility.

Comparing the results of the viability test shown in Figure 1 and those on DNA morphology in Figures 4, 5, and 8 reveals that the kinetics of DNA fragmentation are at their maxi-

mal level at 18–24 h, while the trypan blue exclusion assay is maximal at 48 h. This 24 h delay clearly demonstrates that in general intranuclear events involved in the degradation of DNA occur well before global cellular disintegration; this impression is consistent with kinetics observed in most systems. Therefore, the DNA synthesis activity observed at early time points (0–6 h) may indeed constitute a repair and rescue effort. However, as discussed above, it is too feeble to overcome the massive force inducing fragmentation; by 18 h, most cells exhibit fragmented nuclei, as seen in Figure 3C. Afterwards, cells disintegrate and are no longer sufficiently viable to exclude trypan blue stain. Globally, cells activated for apoptosis may be distinguished by the initial attempt at DNA repair, which is, however, overcome by massive DNA fragmentation and eventual cellular disintegration.

Taken together, our results strongly suggest that cell-cycle traverse and the early events of cell death are partly related and share some similarities in the experience of $G_1$ phase and early S phase. It is, however, obvious that the path leading to programmed cell death (apoptosis) is an abortive one. The divergence of pathways between normal cell-cycle traverse and apoptosis may occur at the $G_1$/S boundary. Our findings lead us to suggest that at least some survival factors may be required for the completion of successful cell-cycle traverse, without which endonuclease is active and DNA fragmentation occurs. The identities of these survival factors, and the gene expressions responsible for the activation of endonuclease activity, are the focus of our future research. The results presented here provide a beginning picture of the kinetic map describing the molecular process of apoptosis in cultured cells, thus facilitating a readily available system, murine 3T3 fibroblasts, where one may investigate unique genes responsible for the block at the $G_1$/S border in cells en route to DNA fragmentation and death.

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