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Engagement of DNA polymerases during apoptosis

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Abstract. DNA replicative and repair machinery was investigated by means of different techniques, including *in vitro* nuclear enzymatic assays, immunoelectron microscopy and confocal microscopy, in apoptotic cell lines such as HL-60 treated with methotrexate, P815 and K562 exposed to low temperatures and Friend cells exposed to ionizing radiation. The results showed a shift of DNA polymerase α and β activities. DNA polymerase α, which in controls was found to be the principal replicative enzyme driving DNA synthesis, underwent, upon apoptosis, a large decrease of its activity being replaced by DNA polymerase β which is believed to be associated with DNA repair. Such a modulation was concomitant with a topographical redistribution of both DNA polymerase α and the incorporation of BrdUrd throughout the nucleus. Taken together, these results indicate the occurrence of a dramatic response of the DNA machinery, through a possible common or at least similar behaviour when different cell lines are triggered to apoptosis. Although this possibility requires further investigation, these findings suggest an extreme attempt of the cell undergoing apoptosis to preserve its nuclear environment by switching on a repair/defence mechanism during fragmentation and chromatin margination.

Apoptosis is a term given in the past to indicate a process resulting in typical morphological changes in the cell and culminating in cell death (Kerr, Wyllie & Currie 1972). It is now known that it represents a regulated process which plays a central role in development and in many physiological and pathological conditions. It occurs in two physiological phases: the entry in a condemned phase (commitment), which is accomplished in a cell-autonomous fashion, followed by a transition to a finally common execution phase, which includes all the known morphological features. These involve both the cytoplasmic and the nuclear compartment (Earnshaw 1995). In the latter, chromatin becomes progressively more condensed and collapses against the nuclear periphery. Nuclear pores redistribute along areas of the nuclear envelope facing the diffuse chromatin (Lazebnik *et al.* 1993), and the lamina disassembles (Ucker *et al.* 1992). Recently, aggregates of nuclear matrix proteins have also been seen in apoptotic cells and have been proposed as a possible hallmark of the apoptotic process (Tinnemans *et al.* 1995, Zweyer *et al.* 1997). In addition, among the biochemical events occurring in the nucleus, also the DNA fragmentation, medited by an as yet unknown Ca^{2+} mediated and Mg^{2+} dependent endonuclease producing 180–200 bp (base pair) oligonucleotides, has long been considered as a typical feature of apoptosis (Neamati *et al.* 1995). Recent

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evidence, however, indicates that it is not always notable in all dying cells (Falcieri *et al.* 1993). Thus, investigations on the nuclear enzymology and reorganization appear to be crucial for the comprehension of the apoptotic cascade. With this in mind, we have focused our attention on the behaviour of the DNA machinery in different cell models triggered to apoptosis by a variety of stimuli. This was accomplished by means of several techniques including confocal microscopy, immunoelectron microscopy, *in situ* nick translation and *in vitro* nuclear enzymatic assays.

MATERIALS AND METHODS

Cell culture and induction of apoptosis

HL-60 human promyielocytic cells, K562 human erythroleukaemia cells, P815 murine mastocytoma cells and Friend murine erythroleukaemia cells were cultured in tissue culture flasks (AS Nunc, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% fetal bovine serum. In all cultures, medium was renewed twice a week. All the cultures were controlled routinely for mycoplasma contamination. For induction of apoptosis cells were treated as follows.

HL-60 cells: HL-60 cells were exposed for times up to 15 h to 10^{-7} mM methotrexate (MTX).
K562 and P815 cells: K562 and P815 cells were incubated for 2 h at 0°C and then for 2 h at 22°C.
Friend cells: Friend cells were irradiated (3 Gy/min for 20 min) at room temperature by a Mevatron 74 Siemens linear accelerator (photonic energy: 10 MeV). Cells were then kept at 37°C for 18 h and, when required, pulsed, during the last hour with 50 μM 5-bromo-2′-deoxyuridine (BrdUrd) (Sigma Chemical Co., St. Louis, MO).

Source of antibodies

The monoclonal antibody to DNA polymerase α (MBL Co., Nagoya Japan) has been described elsewhere (Masaki *et al.* 1982). The monoclonal antibody to BrdUrd (Becton Dickinson, Palo Alto, CA) was as already reported (Rizzoli *et al.* 1992).

Electron microscopy (TEM)

Cells were fixed in 2.5% glutaraldehyde, alcohol dehydrated, epoxy-resin embedded and uranyl acetate–lead citrate stained (Falcieri *et al.* 1994).

Electron microscopy immunocytochemistry

For the immunogold detection of DNA polymerase α, HL-60 cells were fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer for 2 h. Cell pellets were completely dehydrated and embedded in Epon. After etching for 10 min in H_2O_2, thin sections were incubated 10 min at room temperature in TBS blocking buffer (0.05 M Tris HCl, 1% sodium azide and 0.1% bovine serum albumin), pH 7.6. The grids were then incubated with 5% normal goat serum (NGS) for 30 min and reacted overnight with anti-DNA polymerase α monoclonal antibody (1:40) followed by incubation with a goat anti-mouse IgG (Soc. Italiana Chimici, Milan, Italy; 1:13) conjugated with 20 nm colloidal gold for 1 h. The sections were finally stained with aqueous uranyl acetate.

For the detection of BrdUrd incorporation, Friend cells were fixed for 30 min in 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, post-fixed in 1% OsO_4 and embedded in

Spurr resin. Immunocytochemical labelling was performed with the post-embedding immunogold technique as previously reported (Rizzoli *et al.* 1992).

Controls of the procedures consisted of samples not incubated with the primary antibody.

DNA electrophoresis

DNA extraction was carried out with the QIAamp Blood Kit (Quiagen GMBH, Hilden, Germany) following the manufacturer's suggestions. DNA was then analysed on 1% agarose gel.

***In situ* nick translation**

Cells fixed in 3:1 methanol–acetic acid were incubated for 45 min in a solution containing 2 IU of endonuclease-free DNA polymerase I and 10 μM each of dATP, dCTP, dGTP and digoxigenin-11-dUTP in 50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol. After washes in 5% trichloroacetic acid and in Buffer 1 (1 M Tris-buffer, 20 mM $MgCl_2$, pH 7.5), slides were incubated for 20 min at 42°C in Buffer 1 containing 2% bovine serum albumin (BSA), and rinsed with Buffer 1. Nicks were labelled with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody (Boeringher Mannheim, Mannheim, Germany), and nuclei counterstained with 1 μg/ml propidium iodide (PI). Samples were then observed by confocal microscopy. The reactions performed in the absence of DNA polymerase I represented the controls of the procedure and gave constantly negative results.

Immunocytochemical analysis of DNA polymerase α distribution

HL-60 cells, fixed in formaldehyde 3.7% and permeabilized by immersion in PBS 0.1% Triton X-100, were incubated with fluorescein (FITC)-conjugated anti DNA polymerase α monoclonal antibody diluted 1:1 in PBS 20% NGS. After several washes in PBS, samples were mounted in glycerol containing 1 μg/ml PI to counterstain nuclei and observed by confocal microscopy.

Confocal microscopy

Confocal analysis of *in situ* nick translation reaction and of DNA polymerase α distribution was carried out with TCS 4D (Leica, Heidelberg, Germany) mounted on Leitz DMRB (100 $\times$ /1.3 NA oil immersion objective). High resolution fluorescence images were obtained merging the serial optical sections of FITC signal, performed in the z axis, with the corresponding PI images. Acquired images were then elaborated by a 3-D Image Processing System (Leica, Heidelberg, Germany).

Immunocytochemical analysis of BrdUrd incorporation

Friend cells were washed, cytocentrifuged and fixed in 70% ethanol for 30 min at room temperature. For DNA denaturation, the glass slides were incubated with 0.07 M NaOH for 2 min at room temperature and rinsed with PBS; samples were then reacted with mouse MoAb anti-BrdUrd (1:100) and, after washes, with FITC-conjugated goat anti-mouse IgG. Slides were mounted and observed at fluorescence microscopy.

Purification of nuclei

This was accomplished as previously reported using 0.3% Triton X-100 to obtain membrane-depleted nuclei (Miscia *et al.* 1991). Nuclear purity was checked by electron microscopy

observation and by measuring both the glucose-6-phosphatase activity and the recovery of the cytoskeletal marker β tubulin by immunoblotting (Cocco *et al.* 1987, Martelli *et al.* 1996).

In vitro DNA synthesis

Total DNA synthesis and DNA polymerase α and β endogenous activities were assayed as previously reported (Smith & Berezney 1982). DNA polymerase γ activity was assessed by subtracting polymerase α and β activities from the total DNA synthesis.

RESULTS

Hypothermia, methotrexate and radiation produced apoptotic morphology in P815/K562, HL-60 and Friend cells, respectively

The occurrence of most of the typical morphological markers of apoptosis triggered by hypothermia in P815 and K562 cells, by methotrexate in HL-60 cells, and by radiation in Friend erythroleukaemia cells is shown in Figure 1 (a–h). Compared to controls (Figures a, c, e and g), the cells undergoing the different physical and chemical triggers displayed morphological features such as chromatin margination, condensation, and formation of micronuclei (Figures b, d, f and h). The apoptotic typical DNA ladder was detectable in HL-60, P815 and K562 cells (Figure 2) while in irradiated Friend cells it was not notably evident. The evidence of the DNA fragmentation was also studied by means of confocal microscopy performing a three dimensional analysis of the *in situ* nick translation reaction (Figure 3). The nicks occurring during the apoptotic process served as templates for the DNA synthesis catalysed by the DNA polymerase used in the reaction mixture; thus, in the healthy cells, the nucleus appeared completely labelled by the PI fluorochrome (red), while the green/yellow fluorescence of FITC revealed the single stranded DNA produced during apoptosis. Since this event is not a synchronized process, samples displayed different features and topographical distribution of the nicked areas. Indeed, the FITC signal appeared organized either in cup-shaped areas or spotted or homogeneously distributed throughout the nucleus. In addition, the percentage of nicked cells was not identical among the samples, depending on the cell type, on the agent triggering apoptosis, and on the length of the treatment.

Shift of DNA polymerase α and β activities during the apoptotic process

The *in vitro* DNA synthetic machinery was tested in nuclei isolated from both controls and cells undergoing apoptosis by the different triggers as reported in the material and methods (Figure 4). In the controls, DNA synthesis was found to be driven mainly by DNA polymerase α, while the activity of DNA polymerase β was detected to a greatly lesser extent. Low levels of DNA polymerase γ activity was a common feature of such *in vitro* DNA systems. After the different stimuli triggering apoptosis, the activity of DNA polymerase α decreased progressively and dramatically though with different features, possibly depending on the cell type and treatment. Parallel to such an event, the activity of DNA polymerase β evidently increased, becoming the principal activity of all the *in vitro* DNA systems tested. DNA polymerase γ activity did not undergo any substantial changes in P815, HL-60 and Friend cells, while in K562 it was found increased relative to the controls, even though such an increase did not reach the DNA polymerase β levels.

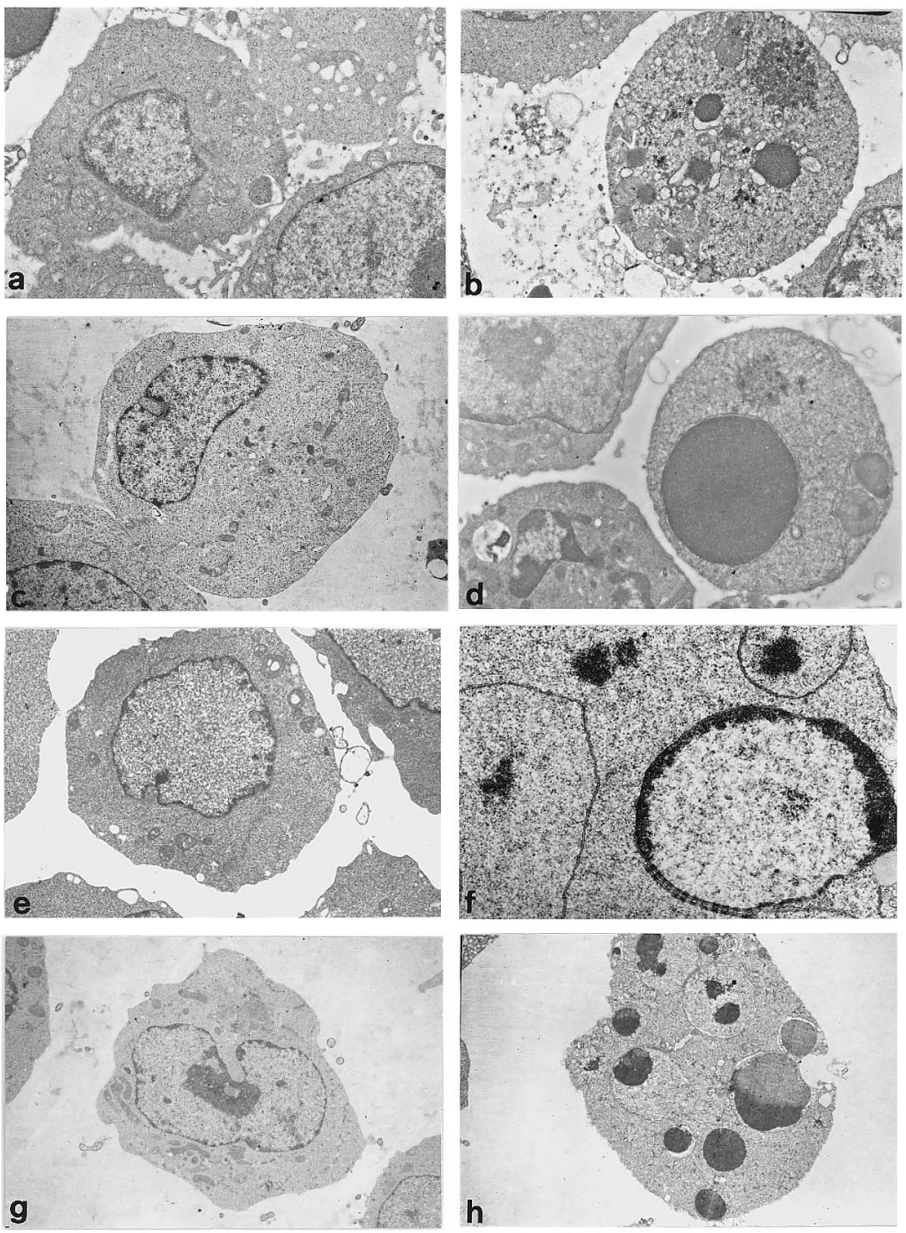

Figure 1. Transmission electron microscopy (TEM) of: (a, b) control and thermal shocked (2 h at 0°C) P815 cells (× 3000); (c, d) control and thermal shocked (2 h at 0°C) K562 cells (× 3000); (e, f) control and MTX treated (15 h) HL-60 cells (e = × 3000; f = × 6500); and (g, h) control and irradiated (3 Gy/min for 20 min) Friend cells (× 3000). Treated cells displayed the stereotypic morphological pattern of apoptosis, such as chromatin condensation and margination along with formation of micronuclei.

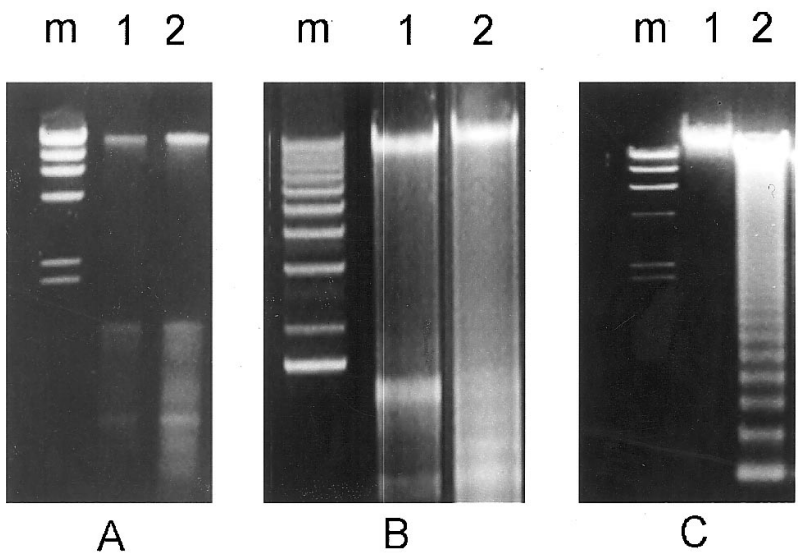

Figure 2. Electrophoretic pattern of DNA from K562 (A) HL-60 (B), and P815 (C) cells. Lane 1, controls; lane 2, treated cells; m, standard markers. In K562 and HL-60 cells the typical DNA apoptotic ladder is accompanied by a smear due to secondary necrosis.

Redistribution of DNA polymerase activities throughout the nucleus

Given the dramatic modulation of the DNA polymerase activities, mostly involving polymerases α and β, the question of a possible redistribution of such enzymes and of their related activities in particular areas of the nucleus arose. Such an analysis was accomplished in HL-60 by means of confocal microscopy and by immunoelectron microscopy using an anti-DNA polymerase α monoclonal antibody. The analysis of the distribution of the enzyme, assessed by confocal microscopy (Figure 5), revealed that it was largely dispersed throughout the nucleus (green fluorescence) in the controls (A), while, upon MTX treatment, it underwent an evident redistribution, being detectable either as distinct clusters or located along the internal periphery of the nucleus like a ring from which beams of fluorescence emerged (B). The immunoelectron microscopy (Figure 6) supported such a picture by showing, relative to the controls (a), the dramatic rearrangement of the DNA polymerase α (gold granules) almost exclusively in the areas of condensed chromatin after MTX treatment (b). A very similar behaviour was found in K562 cells by means of confocal microscopy (not shown), however, because the DNA polymerase α antibody available cross reacts extensively with human but not with rodent α enzymes, we could not perform the same analysis in P815 and Friend cells. A further approach in the investigation of a possible redistribution of the DNA polymerases during apoptosis was the study of the distribution of DNA replicative sites in Friend cells through the BrdUrd–anti-BrdUrd immunofluorescence and immunogold techniques, in light and electron microscopy, respectively. Two distinct patterns of labelling were detected by immunofluorescence (Figure 7): a diffuse fluorescent labelling in the controls (A) and a spotted redistribution throughout the nucleus (B) in irradiated cells. The immunoelectron microscopy (Figure 8) showed a diffuse gold labelling in the control cells (a) and a clear relocation of the label in the areas of condensed chromatin after irradiation (b).

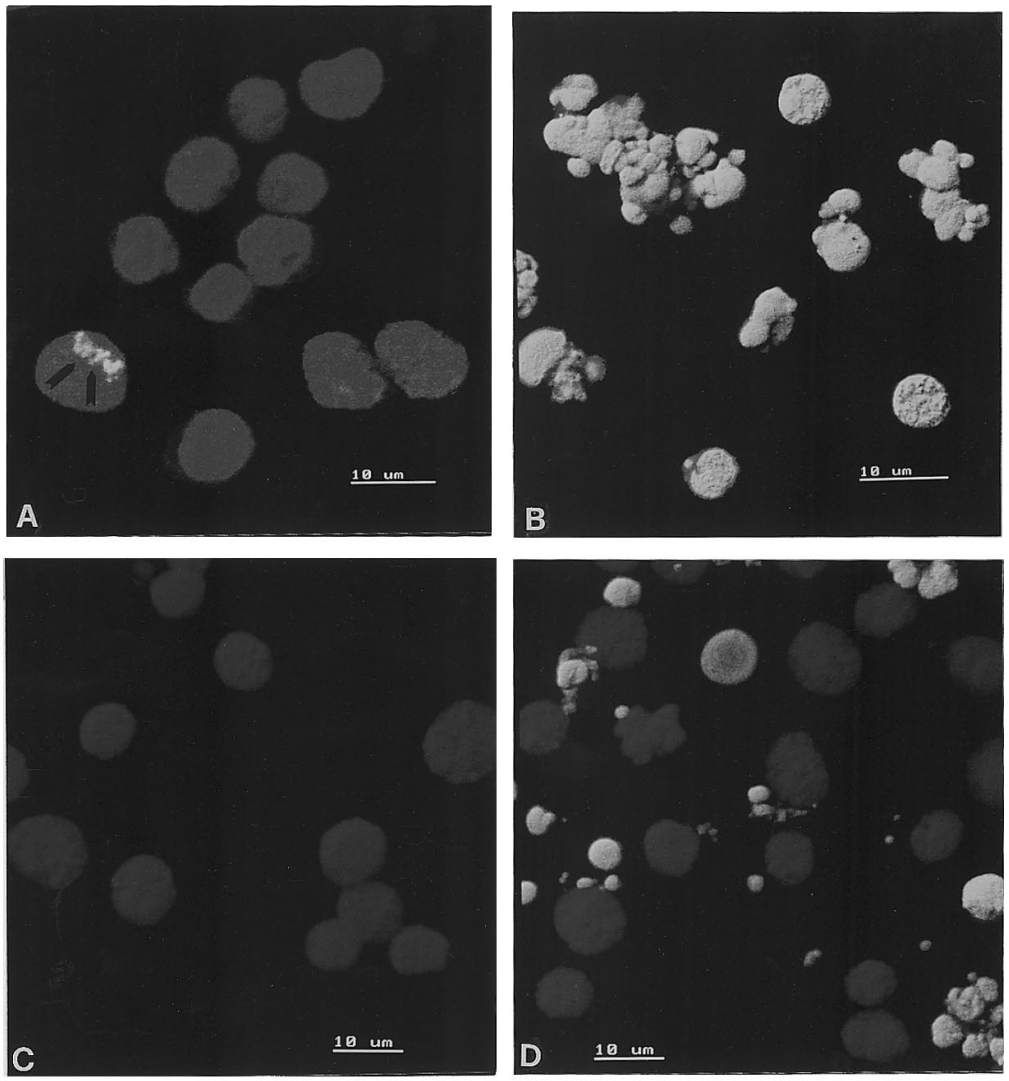

Figure 3. Cofocal microscopy analysis of *in situ* nick translation reaction in HL-60 (A, B), P815 (C, D) and K562 (E, F) cells. The yellow/green fluorescence identifies the areas of cleaved DNA while red PI staining corresponds to intact DNA. (A, C, E), controls; (B, D, F), treated cells. The stochastic nature of the transition from the condemned to the execution phase accounts for the different morphological patterns of the nicked areas. The FITC signal appears organized either in cup-shaped or spotted or homogeneously distributed throughout the nucleus. The three-dimensional analysis allowed us to detect the budding and/or the burst of micronuclei, whose number differed depending on the model investigated, always containing cleaved DNA. The occurrence of spontaneous apoptosis ($<1\%$) in untreated cells is sometimes appreciable (arrowheads).

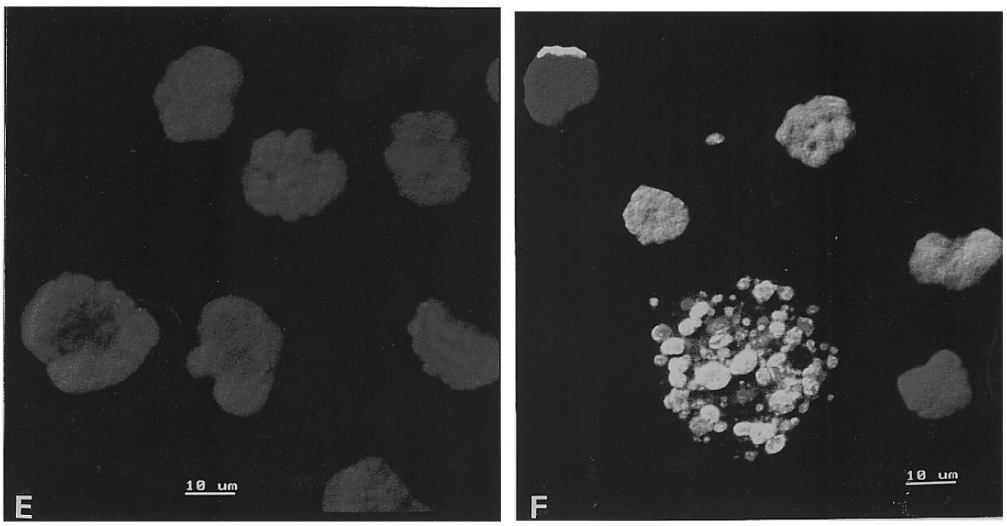

Figure 3. continued

DISCUSSION

Three major types of DNA polymerases referred to as α, β and γ have been reported and largely investigated in eukaryotic cells (Gera *et al.* 1993, Smith & Berezney 1983). Evidence from different laboratories supports the concept that DNA polymerase α is generally implicated in DNA replication, whereas polymerase β is associated with DNA repair after injury. As far as DNA polymerase γ is concerned, different roles for the involvement of its activity have been described in the control of the DNA synthesis. However, it appears to be identical to the polymerase seen in isolated mitochondria. It is worth noting that such enzymes have been demonstrated to be cell cycle specific factors, whose activity would be dynamically controlled during cell growth regulation (Smith & Berezney 1983).

In this investigation we have reported the response of the DNA machinery to various stimuli triggering apoptosis in different cell lines in order to gain an insight into their involvement during the time course of the apoptotic processes. A common response of DNA polymerases α, β and γ was observed during apoptosis though with some slight differences depending possibly on the type of the stimulus and on the mode of treatment. Such a response always included a decrease of DNA polymerase α activity and its replacement by DNA polymerase β activity seen during the time of the apoptotic processes. Since this latter enzyme is very active on nuclear nicked templates, its progressive stimulation upon the induction of the events leading to cell death suggests that DNA repair might be a process activated by the cell in the attempt to rescue its DNA machinery and, in turn, the integrity of its nuclear components. The role of DNA polymerase γ in this context appears to be not clear since an evident involvement of this enzyme in the response to agents triggering apoptosis was found only in K562 exposed to hypothermia. The lack of a similar response of DNA polymerase γ in P815 cells undergoing the same thermal shock would account for a cell-specific reaction which needs further investigation. The finding in HL-60 cells of the redistribution of DNA polymerase α concomitant with DNA polymerase β engagement would suggest that, upon the requirement of repair activity, the margination of the replicative

enzyme at the periphery of the nucleus and in areas of condensed chromatin may represent a commitment to the quiescent phase, paralleled or immediately followed by DNA polymerase β stimulation. When a different approach to the study of such a phenomenon was carried out in Friend cells by using an anti-BrdUrd monoclonal antibody, a picture strongly fitting with the above hypothesis emerged. Indeed, the shift of BrdUrd labelling

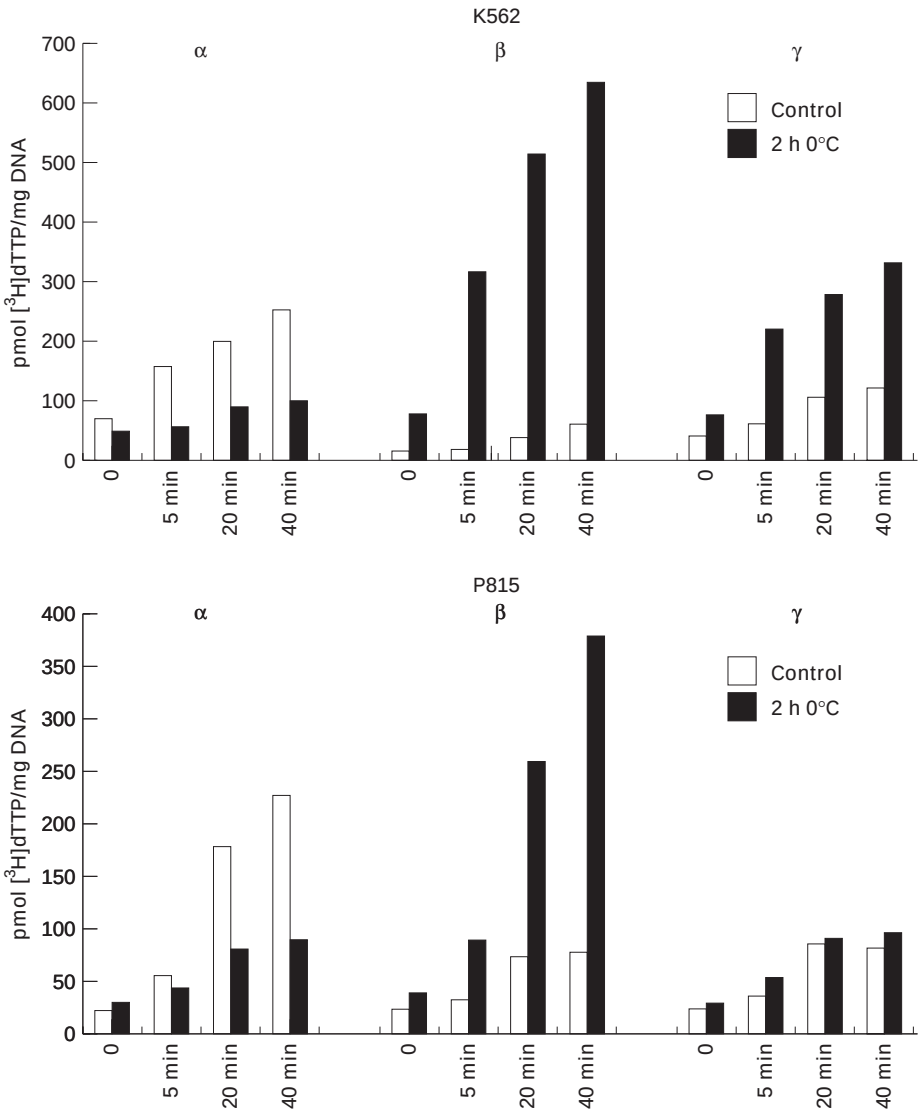

Figure 4. Kinetic assay of DNA polymerase α, β and γ activities. DNA polymerase α appeared to be very effective in control samples, while in the cells undergoing apoptosis DNA polymerase β became the principal enzyme driving the *in vitro* assay. The kinetic analysis of the DNA polymerase γ did not show any significant modulation during the induction of apoptosis process except in K562. Data are the mean of four separate experiments $\pm$SD.

from a diffuse to a spot fluorescence accounts for a general redistribution of the DNA polymerase activities. This result was confirmed by the finding of the aggregation of BrdUrd gold grains in the areas of condensed chromatin at the periphery of the nucleus. Thus, given the decrease of the DNA polymerase α activity, the BrdUrd labelling should relate mainly to polymerase β repair activity. In this context, the use of an anti DNA polymerase β monoclonal antibody, so far to our knowledge not commercially available, would be of great help. Taken together these results indicate the presence of a possible common or at least similar behaviour of the DNA machinery when cells are triggered to apoptosis. The issue of

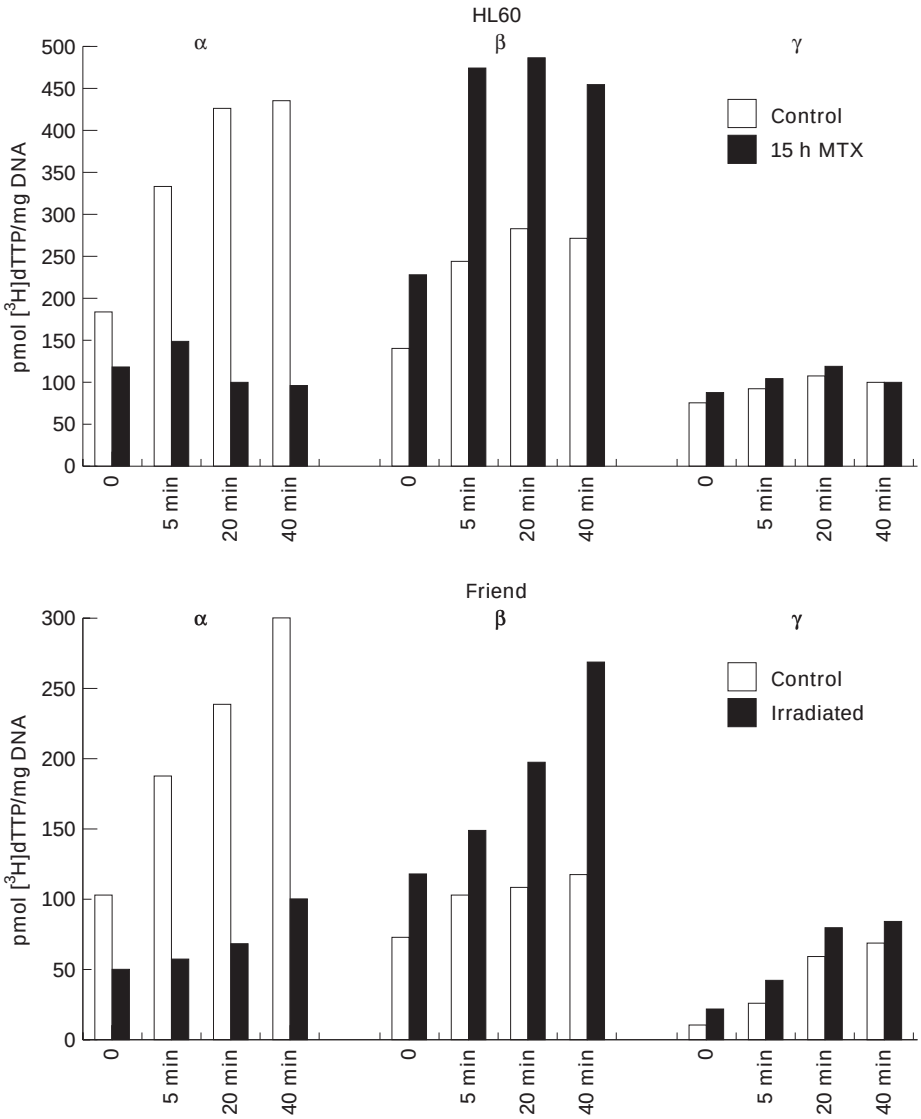

Figure 4. continued

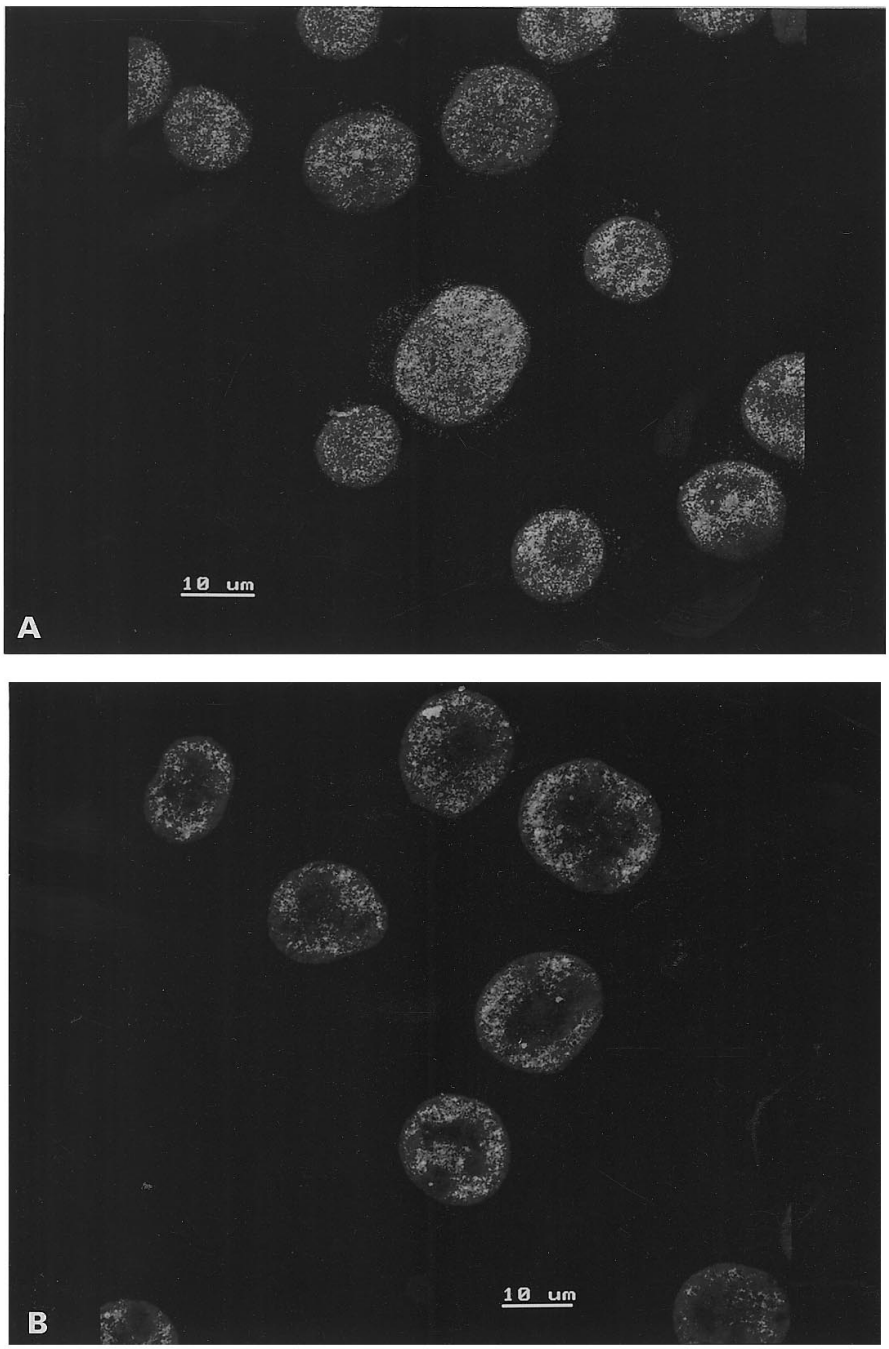

Figure 5. Confocal microscopy analysis of the distribution of DNA polymerase α in control (A) and MTX treated (B) HL-60 cells. The green fluorescence identifies the enzyme which appeared largely dispersed throughout the nucleus (red) in the control cells, while in treated cells it underwent an evident redistribution being notable as clusters or along the internal periphery of the nucleus.

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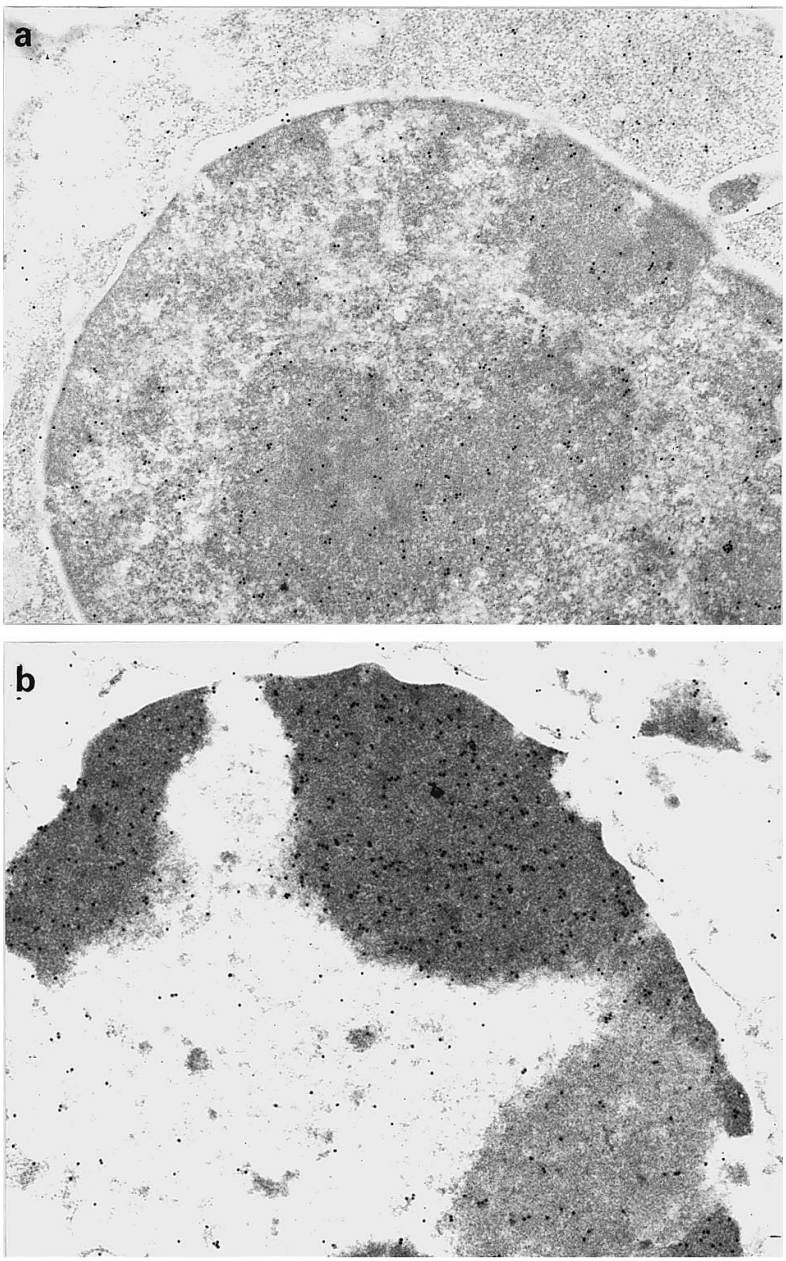

Figure 6. Immunoelectron microscopy of the distribution of DNA polymerase α in control (a) and MTX treated (b) HL-60 cells. Note the shift of the distribution of the enzyme (gold granules) which, relative to the control, after MTX treatment is rearranged mainly in the areas of condensed chromatin ($\times 30\,000$).

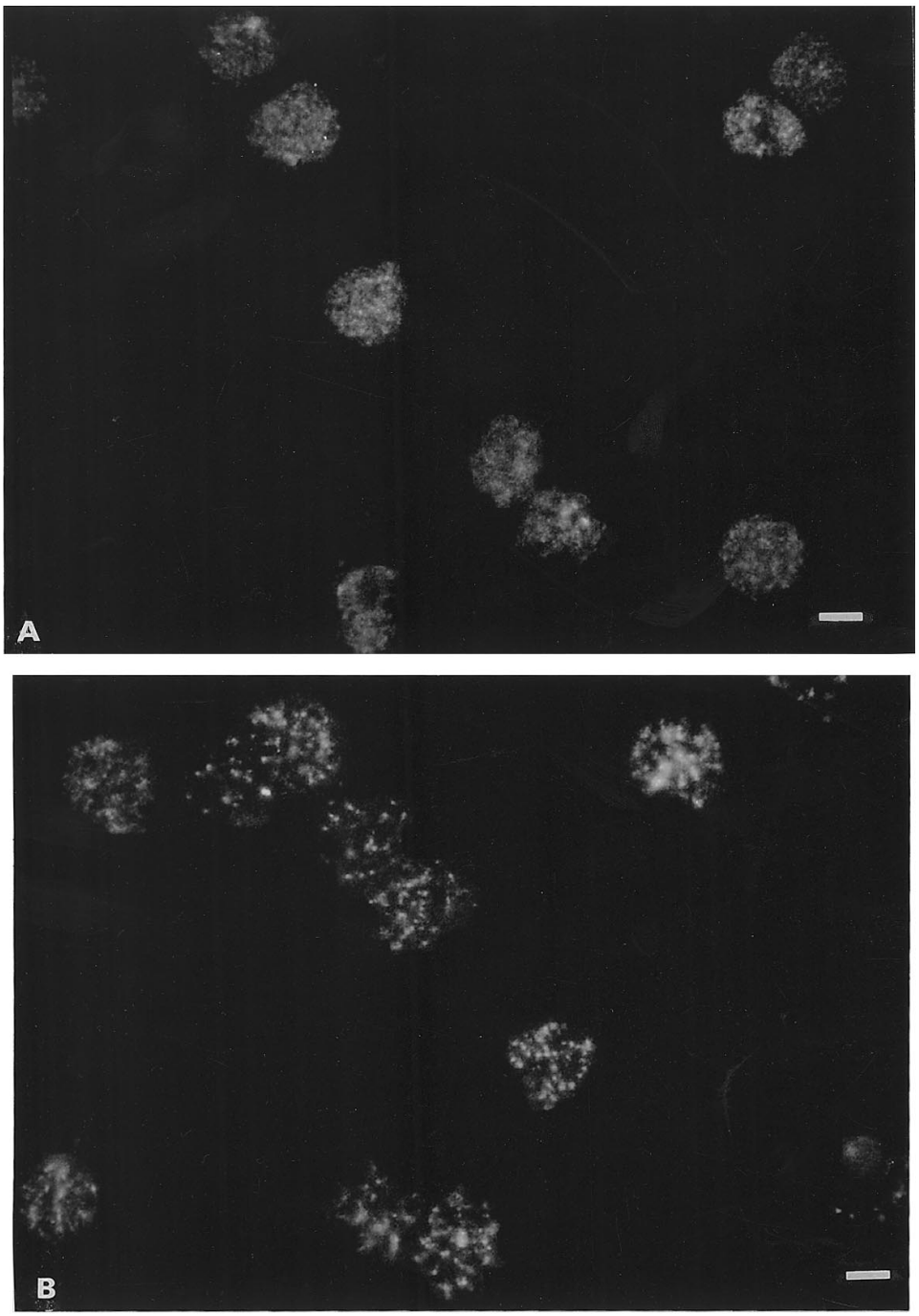

Figure 7. Immunofluorescence analysis of BrdUrd incorporation in control (A) and in irradiated (B) Friend cells. A diffuse fluorescent labelling is detectable in the controls, while a spot-like redistribution is notable in treated cells. Bar = 10 μm.

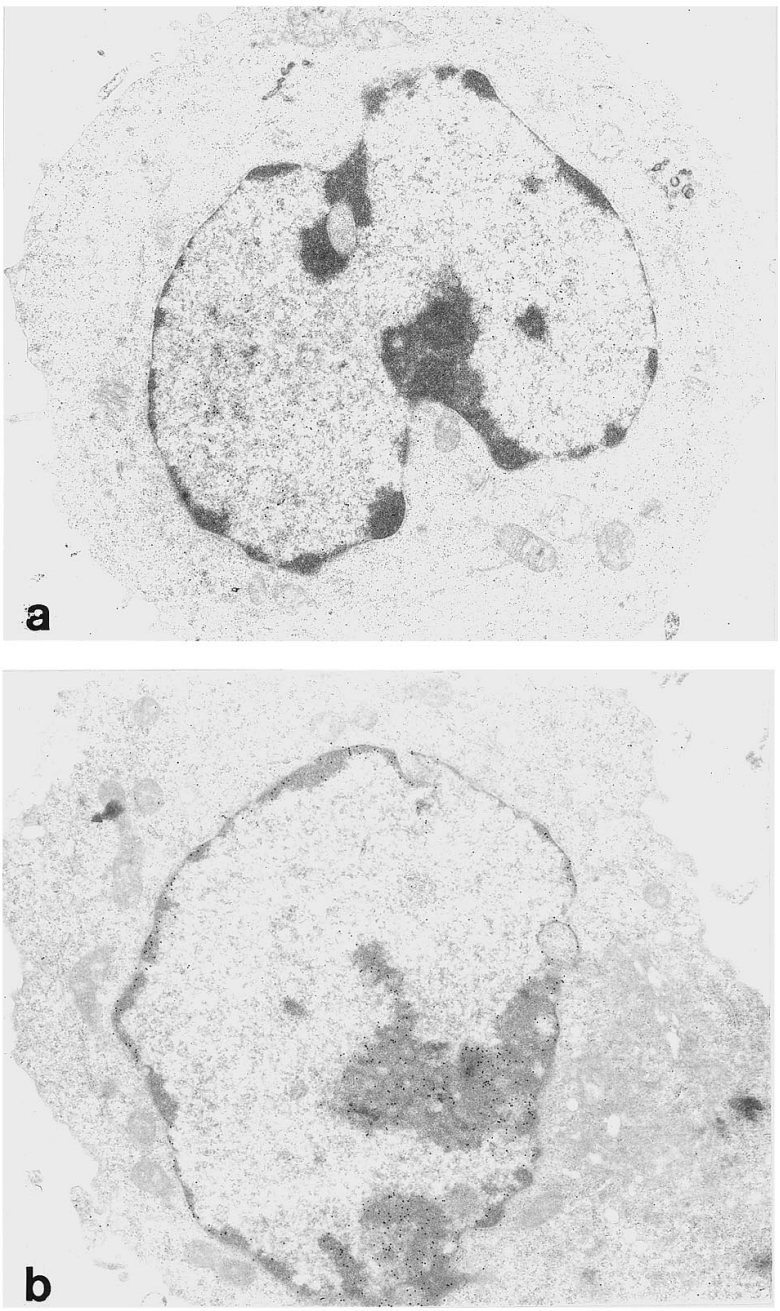

Figure 8. Immunoelectron microscopy of BrdUrd incorporation in control (a) and irradiated (b) Friend cells. Gold granules are regularly dispersed in the nucleus of the controls, while they are evidently relocated in the areas of condensed chromatin after irradiation ($\times$ 20 000).

a typical response sequence during the apoptotic cascade is still an open question, but, even in the presence of some cell-specific features, such a behaviour strongly suggests the possibility that the cell undergoing apoptosis tries to preserve its nuclear environment by switching a repair/defence mechanism. This could contribute to the occurrence of a certain preservation of the plasma membrane and organellar components which surprisingly are seen in advanced stages of the apoptotic event (Wyllie, Kerr & Currie 1980).

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