

# Accelerated Publication

## Nuclear Translocation of Cytochrome *c* during Apoptosis\*

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**Release of cytochrome *c* from mitochondria is a major event during apoptosis. Released cytochrome *c* has been shown to activate caspase-dependent apoptotic signals. In this report, we provide evidence for a novel role of cytochrome *c* in caspase-independent nuclear apoptosis. We showed that cytochrome *c*, released from mitochondria upon apoptosis induction, gradually accumulates in the nucleus as evidenced by both immunofluorescence and subcellular fractionation. Parallel to nuclear accumulation of cytochrome *c*, acetylated histone H2A, but not unmodified H2A, was released from the nucleus to the cytoplasm. Addition of purified cytochrome *c* to isolated nuclei recapitulated the preferential release of acetylated, but not deacetylated, histone H2A. Cytochrome *c* was also found to induce chromatin condensation. These results suggest that the nuclear accumulation of cytochrome *c* may be directly involved in the remodeling of chromatin. Our results provide evidence of a novel role for cytochrome *c* in inducing nuclear apoptosis.**

Apoptosis or programmed cell death is a cellular process used by higher organisms to eliminate unwanted and damaged cells. Events involved in programmed cell death are strictly controlled from embryogenesis to cell adaptation and to tissue remodeling (1). Defect(s) in apoptosis has been linked to various genetic diseases including neurodegenerative disorders, immune deficiency, and tumorigenesis.

The release of cytochrome *c* from mitochondria marks a major event during apoptosis (2–4). Once released into cytosol, cytochrome *c* is known to form a molecular complex with Apaf-1, which then activates caspase cascade leading to expression of many apoptotic events (2–4). Recent studies have also demonstrated a caspase-independent effect of cytochrome *c* during apoptosis. Cytochrome *c* released from mitochondria

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was shown to interact with the  $\text{InsP}_3$ <sup>1</sup> receptor ( $\text{InsP}_3\text{R}$ ) localized in the endoplasmic reticulum (5), which induces the release of calcium from the endoplasmic reticulum and amplifies apoptotic signals. Extensive studies have shown, however, that cells lacking Apaf-1, caspase-3, or caspase-9 can also undergo apoptosis induced by mitochondria dysfunction, suggesting the existence of caspase-independent pathways for cell death (6–11).

Chromatin condensation is one of the hallmarks of apoptosis. But the exact role of mitochondria-released cytochrome *c* in inducing the condensation of nuclear chromatin is unknown. It is believed that caspase-dependent activation of caspase-activated DNase, which leads to DNA fragmentation in cells undergoing apoptosis, is in part responsible for chromatin condensation.

In this study, we examined the time-dependent redistribution of released cytochrome *c* in different subcellular organelles during apoptosis. We observed that cytochrome *c*, once released from mitochondria, accumulated in the cytoplasm followed by gradual accumulation in the nucleus. Concomitant with nuclear migration of cytochrome *c*, acetylated histone H2A, but not bulk histones, was found to exit from the nucleus to the cytoplasm. We also observed that cytochrome *c* was able to influence chromatin condensation *in vitro*. Our data show that nuclear accumulation of cytochrome *c* may be directly involved in the remodeling of chromatin.

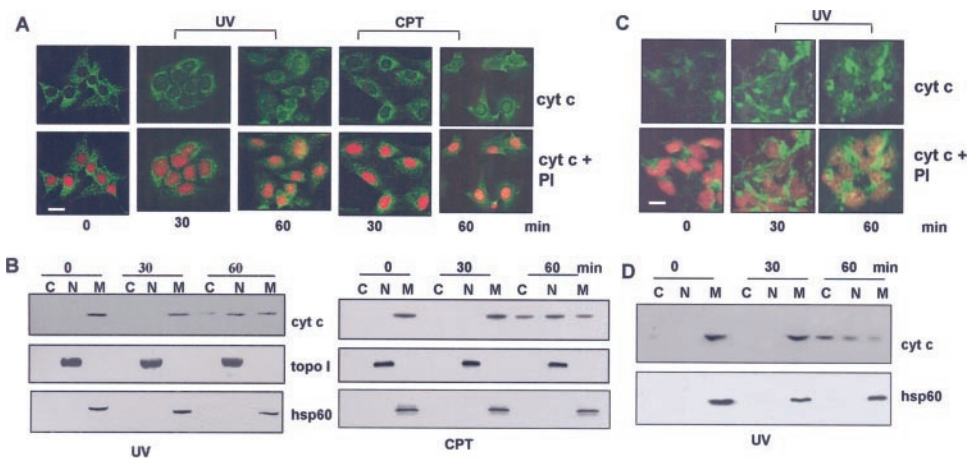
### MATERIALS AND METHODS

**Cell Culture**—HeLa cells were cultured to subconfluence in Dulbecco’s modified Eagle’s medium containing 10% calf serum at 37 °C. HeLa cells were induced to undergo apoptosis by either UV irradiation (20 mJ/cm<sup>2</sup>) using a Stratallinker UV cross-linker (Stratagene, La Jolla, CA) or treatment with camptothecin (CPT) (10 μM). A peptide inhibitor of caspase-3 (Z-DEVD-FMK, Calbiochem) was added to the culture media at a concentration of 50 μM. Cerebellar granule neurons (CGNs) from rat pups were isolated according to the procedure described previously (12).

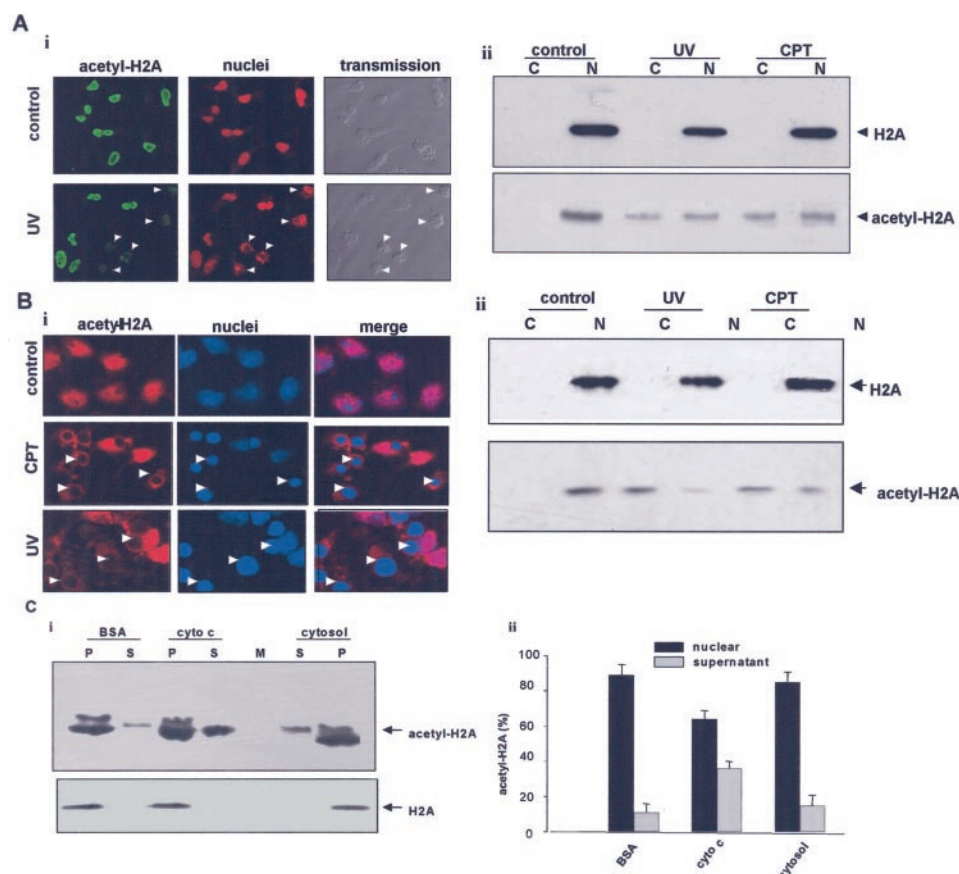
**Immunocytochemistry**—HeLa cells or CGNs grown on glass coverslips were irradiated with UV or treated with CPT. Cells were left for different incubation times in fresh medium, followed by fixation for 20 min at –20 °C in a solution of 95% (v/v) ethanol/5% (v/v) acetic acid. Cells were then treated with blocking buffer (PBS-azide + 10% (v/v) fetal calf serum) for 30 min at room temperature and then incubated with monoclonal anti-cytochrome *c*, or monoclonal anti-acetyl-H2A antibodies, followed by washing in PBS-azide for 5 min and probing with the appropriate secondary fluorescein isothiocyanate- or TRITC/Texas Red-labeled antibody for 2 h. Staining of nuclei was accomplished by incubation with propidium iodide (5 μg/ml) or Hoechst dye (10 μM) for 10 min after the secondary antibody treatment. The slides were then washed extensively in PBS-azide and mounted using vectashield medium (Vector Laboratories, Burlingame, CA). Specimens were viewed using either a Zeiss LSM410 or a Bio-Rad Radiance2100 laser scanning confocal microscope equipped with an argon/krypton lasers or argon/HeNe lasers with excitation at 488 and 568 nm for viewing fluorescein and TRITC, respectively.

**Subcellular Fractionation and Western Blotting**—Separation of cytoplasm from mitochondria and nuclear membrane fractions were performed using established procedures (13). Equal volume of 2× Laemmli SDS sample buffer was added to nuclear, mitochondrial, and cytoplasmic fractions. Samples were analyzed by SDS-PAGE. Western blotting

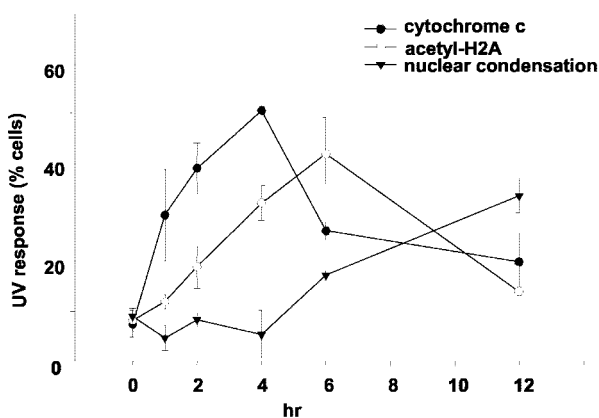
<sup>1</sup> The abbreviations used are:  $\text{InsP}_3$ , inositol 1,4,5-trisphosphate;  $\text{InsP}_3\text{R}$ ,  $\text{InsP}_3$  receptor; CPT, camptothecin; Z, benzoyloxycarbonyl; FMK, fluoromethyl ketone; CGN, cerebellar granule neuron; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; BSA, bovine serum albumin; DAPI, 4’,6-diamidino-2-phenylindole.



**FIG. 1. Ultraviolet light (UV) and camptothecin (CPT) induce nuclear translocation of cytochrome *c*.** Immunofluorescence studies in HeLa cells (A) and cerebellar granule cells (C). HeLa cells or rat (6-day-old pups) cerebellar granule neurons, cultured on glass coverslips, were exposed to UV (20 mJ/cm<sup>2</sup>) or CPT (10  $\mu$ M) for various time periods (0, 30, and 60 min). After fixation, the subcellular distribution of cytochrome *c* was visualized with anti-cytochrome *c* antibodies (green fluorescence) using a confocal microscope. Nuclei were stained with propidium iodide (red fluorescence). Immunoblotting studies of cytochrome *c* distribution in UV-irradiated or CPT-treated HeLa cells (B) and UV-irradiated rat cerebellar granule neurons (D). UV-irradiated and CPT-treated cells were fractionated to yield nuclear (N), mitochondrial (M), and cytosolic (C) fractions. The purity of the subcellular fractions was verified by Western blotting using anti-topoisomerase I and anti-hsp60 antibodies for detecting nuclear- and mitochondrial-specific proteins, respectively. The data are representative of three independent experiments.



**FIG. 2. Cytochrome *c* induces release of acetylated histone from the nucleus.** UV and CPT induce selective release of acetylated histone 2A from the nucleus to the cytoplasm in HeLa cells (A) and rat CGNs (B). *i*, immunofluorescence studies. HeLa cells were exposed to UV (20 mJ/cm<sup>2</sup>), and CGNs were exposed to UV (20 mJ/cm<sup>2</sup>) or CPT (10  $\mu$ M) followed by incubation for 6 h. Immunostaining was performed using anti-acetyl-H2A antibody (green fluorescence for HeLa cells and red for CGNs). Nuclei were stained with propidium iodide (red fluorescence) for HeLa cells and Hoechst (blue fluorescence) for CGN. Arrows point to cells in pre-apoptotic stages, with reduced level of acetyl-H2A and fragmented chromatin structure in the nuclei. *ii*, biochemical fractionation studies. HeLa cells and CGNs were either UV-irradiated or treated with CPT. Biochemical fractionation was performed as described under "Materials and Methods." Specific antibodies against H2A or acetyl-H2A were used in Western blotting. The data are representative of three independent experiments. C, cytochrome *c* induces the release of acetyl-H2A from chromatin *in vitro*. *i*, immunoblotting analysis. Nuclear and cytoplasmic fractions were prepared from untreated HeLa cells as described under "Materials and Methods." Cytochrome *c* (1 mg/ml), cytosol (1 mg/ml), or BSA (1 mg/ml) was added to the nuclear fraction and incubated at 25  $^{\circ}$ C for 30 min. Nuclei (P) were then separated from the supernatant (S) by centrifugation. Western blotting was performed using antibodies against acetyl-H2A or H2A. *ii*, quantitation of the immunoblotting results. The immunoblotting results in *i* were quantified by using the Kodak Imaging Station 2000R.



**FIG. 3. Nuclear accumulation of cytochrome *c*, release of acetyl-H2A from the nucleus, and nuclear condensation in apoptotic cells.** HeLa cells, cultured in glass coverslips, were exposed to UV (20 mJ/cm<sup>2</sup>) and fixed at the indicated times. Cells were immunostained with antibodies against cytochrome *c* or acetyl-H2A and viewed under a fluorescent microscope. Cells were scored for nuclear localization of cytochrome *c*, release of acetyl-H2A from the nucleus, and chromatin condensation. About 200 cells were randomly selected and counted for each of these processes. The results are averages of four independent experiments.

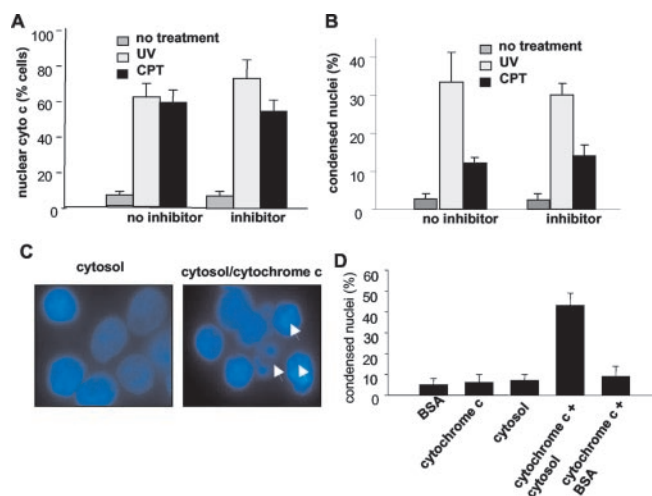
was performed according to the ECL protocol provided by the suppliers (Amersham Biosciences) using specific antibodies. Fractionation of the mitochondrial and nuclear proteins was confirmed by probing the membrane for hsp60 (a mitochondrial protein) or topoisomerase I (a nuclear protein) using their specific antibodies.

**In Vitro Assay of Cytochrome *c*-induced Release of Acetyl-H2A from the Nucleus—**HeLa cells (10<sup>7</sup>) were cultured to semiconfluence. Nuclear, mitochondrial, and cytoplasmic fractions were prepared according to the procedure described above. Nuclei (pellet) were suspended in buffer H (10 mM HEPES, pH 7.0, 50 mM NaCl, 20% (v/v) glycerol, 40 mM  $\beta$ -glycerophosphate, 2 mM MgCl<sub>2</sub>, and 5 mM dithiothreitol) (14). Cytochrome *c* (Sigma), bovine serum albumin (Fraction 8, Sigma), or cytosolic fraction (from untreated cells) was added to the suspended nuclei (10<sup>6</sup> nuclei in 100  $\mu$ l) to a final concentration of 1 mg/ml. Samples were then incubated for 30 min at 25 °C with mild shaking, followed by centrifugation in an Eppendorf microcentrifuge at 2,000 rpm for 10 min at 4 °C. The pellet (nuclei) was suspended in 100  $\mu$ l buffer H. Equal volume of 2 $\times$  Laemmli SDS sample buffer was added to the pellet and supernatant. Western blotting was performed as described above using antibodies against acetyl-H2A (Santa Cruz Biotechnology, Inc. and Cell Signaling Technology) or H2A (Santa Cruz Biotechnology, Inc.).

**In Vitro Chromatin Condensation Assay—**Nuclei were prepared from untreated HeLa cells using the protocol described above and suspended in buffer H. To the nuclear suspension cytochrome *c*, bovine serum albumin (BSA), or a cytoplasmic fraction from UV-irradiated HeLa cells was added. Samples were then incubated for 30 min at 25 °C with mild shaking. Samples were transferred to polylysine (1 mg/ml)-coated glass slides and fixed in 95% ethanol/5% acetic acid at -20 °C for 20 min. Nuclei were then stained with DAPI (10 ng/ml). Chromatin condensation was viewed under a fluorescent microscope.

## RESULTS AND DISCUSSION

**Nuclear Accumulation of Cytochrome *c* during Apoptosis—**DNA damage has been shown to induce apoptosis by releasing cytochrome *c* from mitochondria (8–10). Immunostaining of HeLa cells treated with UV (20 mJ/cm<sup>2</sup>) or CPT (10  $\mu$ M) revealed a time-dependent release of cytochrome *c* from mitochondria to the cytoplasm, followed by accumulation of cytochrome *c* in the nucleus (Fig. 1A). Cytochrome *c* released from mitochondria was found to become visible in the nucleus within 30 min of treatment. Subcellular fractionation also demonstrated a time-dependent migration of cytochrome *c* from mitochondria to the cytoplasm, and then to the nucleus, after either UV irradiation or treatment with CPT (Fig. 1B). Nuclear translocation of cytochrome *c* was also confirmed by its co-fractionation with DNA topoisomerase I (Fig. 1B), a nuclear-specific protein. To test whether cytochrome *c* translocation



**FIG. 4. Nuclear translocation of cytochrome *c* and chromatin condensation are independent of caspase-3 activation.** HeLa cells were cultured to about 40% confluence in Dulbecco's modified Eagle's medium containing 10% calf serum and then pretreated with the caspase-3 inhibitor (Z-DEVD-FMK) (50  $\mu$ M) for 1 h. Pretreated HeLa cells were then either UV-irradiated (20 mJ/cm<sup>2</sup>) or treated with CPT (10  $\mu$ M). After 4 h (for cytochrome *c* staining, see A) or 12 h (for chromatin condensation, see B) of incubation, cells were fixed and stained for cytochrome *c* using anti-cytochrome *c* antibody (Santa Cruz Biotechnology, Inc.) or for DNA (DAPI staining). Cells showing nuclear translocation of cytochrome *c* (A) and chromatin condensation (B) were scored from random fields under a fluorescent microscope as described under "Materials and Methods." C, cytochrome *c*-induced chromatin condensation. Nuclear fraction was prepared from untreated HeLa cells, and cytoplasmic fraction (cytosol) was prepared from UV-irradiated HeLa cells as described under "Materials and Methods." Cytochrome *c* (1 mg/ml), cytosol (1 mg/ml) from UV-treated cells, BSA (1 mg/ml), cytochrome *c* plus cytosol, or cytochrome *c* plus BSA was added to the nuclear fraction and incubated at 25 °C for 30 min. Nuclei were then fixed and stained with DAPI (10 ng/ml). Chromatin condensation was viewed under a fluorescent microscope. *Arrows* show condensed nuclei. Percent of condensed nuclei in each sample is shown in D.

during apoptosis is a general phenomenon, we have performed a similar study in post-mitotic (rat) CGNs treated with either CPT or UV. We have observed nuclear translocation of cytochrome *c* in CGNs with a similar kinetics (Fig. 1, C and D). These results provide the first evidence for nuclear accumulation of cytochrome *c* in cells undergoing apoptosis.

**Nuclear Translocation of Cytochrome *c* Induces Release of Acetyl-H2A—**Cytochrome *c* is known to bind DNA and may thus affect the chromatin structure upon its translocation into the nucleus. Using confocal microscopy, we found that the acetylated form of H2A, but not bulk H2A, was released from the nucleus into the cytosol after UV irradiation in HeLa cells (Fig. 2A). The specific release of acetyl-H2A, but not unmodified H2A, into the cytoplasm was further confirmed by biochemical fractionation and Western blotting analysis. As shown in Fig. 2A, 6 h after exposure to UV or CPT, about 30–40% of acetyl-H2A was released from the nucleus to the cytoplasm. In contrast, there was no detectable level of acetyl-H2A in the cytoplasm in untreated cells. We have further studied whether acetylated form of H2A, but not unmodified H2A, releases from the nucleus to the cytoplasm in rat post-mitotic neurons. Like HeLa cells, we have found that acetylated H2A released from the nucleus to the cytoplasm in neurons treated with either CPT or UV with a similar kinetics (Fig. 2B). These results suggest that apoptotic stimuli-induced release of acetyl-H2A from the nucleus is not specific to a particular cell line.

The temporal correlation between nuclear migration of cytochrome *c* and release of acetylated H2A into the cytoplasm suggests a potential causal relationship. In view of the known



DNA affinity for cytochrome *c*, we have studied if cytochrome *c* can directly displace acetyl-H2A from chromatin in isolated nuclei. We found that addition of cytochrome *c* to isolated nuclei induced the release of acetyl-H2A but not H2A (Fig 2C). This result supports the notion that cytochrome *c* induces the release of acetyl-H2A from the nucleus possibly by a direct displacement reaction.

Previous studies have demonstrated that induction of apoptosis causes release of core histones into the cytoplasm (15–19). However, in our cultured cell system, no release of core histones was detectable even after 24-h post-induction of apoptosis (data not shown). It seems possible that released of core histones into the cytoplasm is either significantly delayed or absent in our system and is probably carried out by a distinct pathway.

*The Time Course of Nuclear Translocation of Cytochrome c, Release of Acetyl-H2A, and Chromatin Condensation during Apoptosis*—One potential role for cytochrome *c*-induced release of acetylated histone H2A during apoptosis is to inactivate the genetic activity of chromatin by causing chromatin condensation. We have thus studied the time course of accumulation of cytochrome *c* in the nucleus, release of acetylated-H2A into the cytoplasm, and chromatin condensation (Fig. 3). HeLa cells were UV-irradiated and incubated for various time periods. Cells were then fixed, stained, and viewed under a microscope. The appearance of cytochrome *c* in the nucleus and disappearance of acetyl-H2A from the nucleus were detected with specific antibodies against cytochrome *c* and acetyl-H2A, respectively. Chromatin condensation was visualized with propidium iodide (Molecular Probes). As shown in Fig. 3, cytochrome *c* started accumulating in the nucleus within an hour after UV irradiation. After 4 h, about 50% cells had accumulated cytochrome *c* in their nuclei. Parallel to cytochrome *c* accumulation in the nucleus, acetyl-H2A was found to be released into the cytoplasm following a similar time course. By contrast, chromatin condensation was delayed for several hours. After 12 h, about 35% of cells exhibited chromatin condensation. These results suggest that the nuclear translocation of cytochrome *c* and the release of acetyl-H2A precede chromatin condensation.

*Nuclear Translocation of Cytochrome c Is Independent of Caspase Activation*—To study the role of caspase activation on the nuclear translocation of cytochrome *c*, we pretreated the cells with a peptide inhibitor of the caspase-3 (Z-DEVD-FMK). Cells were then UV-irradiated or treated with CPT for 4 h, followed by immunofluorescence examination for nuclear translocation of cytochrome *c*. Cytochrome *c* was found to be translocated into the nucleus in 50–60% of treated cells. Nuclear translocation of cytochrome *c* was unaffected by treatment with the caspase-3 inhibitor (Fig. 4A). Similarly, cells were also analyzed for chromatin condensation after 12 h of UV or CPT treatment. Chromatin condensation, like cytochrome *c* nuclear translocation, was largely unaffected by treatment with the caspase-3 inhibitor (Fig. 4B). These results suggest that both nuclear translocation of cytochrome *c* and chromatin condensation are independent of caspase activation and could be functionally linked. We have thus studied the role of cytochrome *c* on chromatin condensation by treating isolated nuclei with cytochrome *c*. As shown in Fig. 4, C and D, treatment of nuclei with cytochrome *c* did not induce chromatin condensation. Treatment of nuclei with a cytosolic extract isolated from UV-irradiated cells also failed to induce chromatin condensation. However, treatment of nuclei with both cytochrome *c* and a cytosolic extract from UV-irradiated cells induced chromatin condensation in a significant fraction of nuclei (about 35%). These results suggest a potential role of cytochrome *c* in chromatin condensation. However, other factors are clearly involved. Previous studies of chromatin condensation have sug-

gested that peripheral chromatin condensation (stage I) requires the translocation of the apoptosis-inducing factor from mitochondria to nuclei (20, 21). A more advanced pattern of chromatin condensation (stage II) has been shown to require activated caspase-3 or caspase-activated DNase (22–25). The appearance of the condensed chromatin in cytochrome *c*/cytosolic extract-treated nuclei suggests the possibility that cytochrome *c* may play a role in stage II chromatin condensation. Clearly, more studies are necessary to establish the precise role of cytochrome *c* in nuclear apoptosis.

Our results suggest that cytochrome *c* may play multiple roles in addition to activation of capases during execution of apoptosis in the cell. Consistent with this hypothesis, a recent study has demonstrated that cytochrome *c* released from the mitochondria binds to InsP<sub>3</sub>R that is localized in the endoplasmic reticulum (5). Cytochrome *c* released from the mitochondria blocks calcium-dependent inhibition of InsP<sub>3</sub>R function, alters calcium release from the endoplasmic reticulum, resulting in augmented cytochrome *c* release, and amplifies apoptosis signal. Our observation of cytochrome *c*-induced release of acetyl-histone from the nucleus suggests another potential role for cytochrome *c* during nuclear apoptosis. It seems plausible that chromatin remodeling caused by nuclear cytochrome *c* accumulation may impact on genetic activity. Clearly, further studies are necessary to reveal the significance of the nuclear translocation of cytochrome *c* during apoptosis.

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